



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b>  <b>C12N 15/86</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/51809</b>  <b>(43) International Publication Date:</b> 19 November 1998 (19.11.98)
<b>(21) International Application Number:</b> PCT/US98/09553  <b>(22) International Filing Date:</b> 11 May 1998 (11.05.98)  <b>(30) Priority Data:</b> 08/854,601      12 May 1997 (12.05.97)      US  <b>(71) Applicant:</b> UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION [US/US]; 911 William Pitt Union, Pittsburgh, PA 15260 (US).  <b>(72) Inventors:</b> GLORIOSO, Joseph, C.; 202 Scarborough Drive, Cheswick, PA 15024 (US). KRISKY, David; 1551 Field Club Drive, Pittsburgh, PA 15237 (US).  <b>(74) Agents:</b> KILYK, John, Jr. et al.; Leydig, Voit & Mayer, Ltd., Suite 4900, Two Prudential Plaza, 180 North Stetson, Chicago, IL 60601-6780 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> MULTIGENE VECTORS DERIVED FROM HSV		
<b>(57) Abstract</b>  <p>The present invention provides a method for preparing viral vectors. The method comprises co-transfecting a source vector and a mutating cassette together into a population of appropriate host cells, such that homologous recombination occurs between the mutating cassette and the source vector whereby the mutating cassette replaces a region of the HSV genome. The mutating cassette has a unique restriction site not present in the sequence of the vector. The method further comprises plaquing the co-transfected host cells, selecting plaques in which recombination has occurred between the source vector and the mutating cassette, and isolating the viral DNA from the plaques. The isolated viral DNA is digested with a restriction endonuclease appropriate for cleaving the viral DNA at the unique restriction site within the mutating cassette to produce two viral polynucleotides. Following purification, the two viral polynucleotides can be joined to form a viral vector comprising the two viral polynucleotides. Alternatively, the two isolated viral polynucleotides can be joined with an insertion cassette to form a viral vector comprising the insertion cassette at the former locus of the unique restriction site. The present invention further provides a mutant vector, particularly an HSV vector constructed in accordance with the method for the present invention. The present invention further provides a multigene HSV vector, particularly a multigene HSV vector for cancer therapy.</p>		

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## MULTIGENE VECTORS DERIVED FROM HSV

**STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER  
FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT**

5 This invention was made with Government support under Grant Number  
DAMD17-94-J-4039 awarded by the United States Army Postdoctoral Training  
Program in Breast Cancer Biology, and under Grant Numbers RO1-DK49095-  
01(JCG), GM-43534-14(JCG), and RO1E411528(DJF) awarded by the United  
States National Institutes of Health and the Department of Veterans' Affairs. The  
10 United States Government may have certain rights in this invention.

**TECHNICAL FIELD OF THE INVENTION**

The present invention relates to a method for preparing viral vectors and to  
multigene HSV vectors.

**BACKGROUND OF THE INVENTION**

15 Gene transfer technology has wide-ranging utility in a number of  
applications relating to biological research and the treatment of disease. Central to  
this technology is a vector for introducing expression cassettes into target cells  
20 such that the cassettes can be expressed in the target cells. Examples of such  
vectors include naked DNA vectors (such as plasmids), viral vectors (such as  
adeno-associated viral vectors) (Berns et al., *Annals of the New York Academy of  
Sciences*, 772, 95-104 (1995)), adenoviral vectors (Bain et al., *Gene Therapy*, 1,  
S68 (1994)), herpesvirus vectors (Fink et al., *Ann. Rev. Neurosci.*, 19, 265-87  
25 (1996)), packaged amplicons (Federoff et al., *Proc. Nat. Acad. Sci. USA*, 89, 1636-  
40 (1992)), papilloma virus vectors, picornavirus vectors, polyoma virus vectors,  
retroviral vectors, SV40 viral vectors, vaccinia virus vectors, and other vectors.  
Once a given type of vector is selected, its genome must be manipulated for use as  
a background vector, after which it must be engineered to incorporate exogenous  
30 polynucleotides.

A particularly attractive vector system employs recombinant herpes  
simplex virus (HSV) vectors. HSV attacks the human nervous system after a  
primary infection of the dermal or mucosal tissues. HSV naturally enters axonal  
portions of sensory ganglia and is transported to the somas of the cells, whereupon  
35 the viral DNA is released to the nucleus (Stevens, *Microbiol. Rev.*, 53, 318-32  
(1989); Roizman & Sears, in *Field's Virology*, 2d ed. Raven Press, Fields et al.,  
eds. 1795-1841 (1990)). At that point, the wild-type virus either initiates a lytic

cycle characterized by viral replication, or enters a latent phase. During the lytic phase, HSV gene expression proceeds through a well-characterized cascade typified by three discrete phases: Immediate Early (a or IE), Early (b or E), and Late (g or L). Recombinant HSV viruses can be induced to enter latency by  
5 inactivating immediate early genes required for replication (e.g., d120, DeLuca et al., *J. Virol.*, 56, 558 (1985)). During the latent phase, the virus persists in an episomal form for the life of its host (Mellerick and Fraser, *Virology*, 158, 265-75, (1987); Rock and Fraser, *J. Virol.*, 55, 849-52, (1985)), neither interfering with neuronal function nor inducing any autoimmune response (Ramakrishnan et al., *J.*  
10 *Virol.*, 68, 1864-70 (1994); Fruh et al., *Nature*, 375, 415 (1995)).

The HSV viral genome is well characterized, as is its life cycle, and the functions of more than 80 native coding polynucleotides are largely defined. HSV coding polynucleotides are generally contiguous linear sequences, thus facilitating genetic engineering of mutant vectors. Furthermore, as roughly half of the viral  
15 genes are dispensable for growth in cell culture, the possibility exists of deleting large segments of the HSV genome to accommodate transgenic material (Roizman & Sears, *supra*; Glorioso et al., in *Viral Vectors*, Academic Press, New York (Kaplitt & Loewy, eds.) 1-23 (1995)). Theoretically, up to 30 KB of the HSV genome can thus be replaced with exogenous material without requiring  
20 complementary host cells for propagation of the virus.

As the HSV genome is so well characterized, its genome is readily manipulated for use as a background vector. For example, it is possible to produce vectors deficient for essential loci as long as the missing translation product is otherwise provided, such as via a helper virus or complementary cell line. In fact,  
25 of the IE loci (ICP0, ICP4, ICP22, ICP27, and ICP47), only two (ICP4 and ICP27) are required for viral replication. In the absence of these loci, only other immediate early loci are expressed, and the virus is rendered replication-incompetent in non-complementing cell lines. A replication-deficient HSV virus can be induced to exist in host neural tissue in its persistent latent phase without  
30 any apparent pathological effect on the host (Ramakrishnan et al., *supra*), and defective HSV vectors can persist in a similar state in non-neuronal cells as well.

HSV vectors deficient for only one essential IE locus remain highly cytotoxic to infected cells, largely due to expression of the remaining IE gene products (DeLuca et al., *J. Virol.*, 56, 558-70 (1985); Johnson et al., *J. Virol.*, 66,  
35 2952-65 (1992)). However, further research has indicated that mutants deleted for ICP4, ICP22, and ICP27 demonstrate reduced cytotoxicity over singly deficient vectors (see, e.g., Wu et al., *J. Virol.*, 70(9), 6358-69 (1996)). Furthermore, the

deletion of other loci reduces the cytotoxicity of HSV viruses (e.g., ICP47 (including U<sub>S</sub>10-11), UL41, VP16, UL24; see, e.g., Jacobson et al., *J. Virol.*, 63(4), 1839-43 (1989); Johnson, *J. Virol.*, 68(10), 6347-62 (1994)). As such, multideficient HSV vectors represent a desirable choice for gene transfer technology.

Of course, production of multideficient viruses is one step in developing vectors for expression cassette transfer. The second step is engineering a vector containing exogenous expression cassettes. In many applications of gene transfer technology, the expression of multiple transgenes within the target cells is desirable. For example, coordinate expression of multiple cytokine sequences together with a sequence encoding an activator for an antitumor pro-drug is a potentially effective cancer therapy. Preferably, a vector is engineered such that the multiple transgenes are independently controlled (e.g., each is under regulatory control of a separate promoter) to optimize the expression kinetics. Because replication-deficient HSV vectors can transiently or constitutively express exogenous expression cassettes (Fink et al., *Ann Rev. Neurosci.* 19, 265-287 (1996)), HSV vectors containing multiple exogenous cassettes represent powerful agents for gene transfer applications.

Although attractive choices for gene transfer technology, HSV vectors have not been widely utilized for these therapies as of yet, primarily due to the difficulty in engineering vectors using standard procedures. The standard method for engineering mutant HSV viruses is to cotransfect host cells with the source virus and a polynucleotide comprising the desired mutation flanked by regions homologous to the target site within the HSV genome. Within the host cell, homologous recombination produces desired mutant HSV viruses less than 5% of the time, with the efficiency generally proportionate to the size of the flanking regions. Aside from inherently low efficiency, recombinant viruses often grow at markedly reduced rates vis-à-vis unmodified parental viruses within host cells, and so are easily overgrown. Thus, where the source vector is already deficient for native loci (particularly essential loci) viral growth can be substantially compromised, and the efficiency of recombination reduced accordingly. Therefore, screening plaques for desired recombinants is a laborious process, and the process becomes incrementally more tedious with multideficient HSV vectors, especially so where the desired mutation is not readily selectable.

Methods for increasing the efficiency of recombination have been attempted, but each presents significant drawbacks for quickly developing novel transfer vectors. Site-specific recombinases such as the cre-lox recombination

system (reviewed in Kilby et al. *Trends in Genetics*, 9, 413-21 (1993)), have been employed to facilitate introduction of exogenous material into viral genomes (see, e.g., Gage et al., *J. Virol.*, 66, 6509-15 (1992)). By these methods, exogenous polynucleotides are introduced into a recombinase recognition site previously engineered into the HSV genome. Recombinants can be selected by assaying for a reporter construct also present within the cassette. Furthermore, source viruses in which the recombinase recognition site is in a locus conferring growth benefits (e.g., the tk locus) do not enjoy the growth advantages over recombinants as seen in traditional methods. Thus, recombinase-mediated production of vectors is a more efficient method for producing HSV vectors than the co-precipitation method, routinely producing desired recombinants roughly 10% of the time, and in some instances site-specific recombination can be significantly more efficient (Rasty et al., *Meth. Mol. Genet.*, 7, 114-30 (1995)). Despite the gain in efficiency, recombinase-mediated production of vectors presents two significant drawbacks. The first is that the method necessarily incorporates the entirety of any plasmid containing the desired insertion sequence, generally a bacterial plasmid for cloning a desired exogenous cassette. Of course, this requirement partially obviates the advantage inherent in the HSV genome's potentially large capacity to accommodate foreign DNA. Secondly, and more significantly, because the recombinase recognition site is retained (in duplicate) within a recombinant vector, subsequent rounds of site-specific recombination greatly disrupt the vectors and can result in randomization of the genome. Thus, site-specific recombination is not a preferred method for generating HSV vectors comprising multiple transgenes.

In order to enhance the efficiency of vector production, some attempts have been made to introduce unique restriction sites into the HSV genome. For example, in one system, all of the XbaI sites within the HSV genome are removed, and the RSAI site in the unique short region of the genome is changed to an XbaI site (Rixon & McLauchlan, *J. Gen. Virol.*, 71, 2931-39 (1990)). Similarly, another system involves the introduction of a unique PacI site within the LAT region of an HSV mutant lacking 4.1 KB in one LAT copy (i.e., the HFEM mutant) (Huang et al., *Gene Therapy*, 1, 300-06 (1994)). With either of these systems, double-digestion of source vector DNA with the appropriate restriction enzyme permits efficient insertion of cassettes. Furthermore, screening of recombinants is achieved using a selectable marker (i.e., b-galactosidase), which increases overall efficiency. However, these methods are limited to introducing exogenous DNA only at these loci, and generally only permit introduction of single cassettes.

Engineering HSV vectors to include multiple cassettes has proven difficult. Not only are the aforementioned methods inefficient for developing multigene HSV vectors, but certain properties of the HSV genome have thwarted attempts to create multiple insertions using standard means. For example, during replication, the HSV virus reacts to foreign DNA inserted at or near repeat elements. In some cases, a single expression cassette can be effectively introduced, but when the insertion of a second cassette is attempted, the virus reacts by recombining and shuffling its genome, substantially destabilizing the vector. In other instances, insertion of exogenous DNA disrupts viral function as well. Where multiple cassettes contain homologous regions to each other (i.e., a similar promoter, similar polyadenylation sequences, etc.), intragenomic recombination also can occur to destabilize the vector.

In view of the foregoing, there exists a need for a method for efficiently producing multideficient HSV vectors, especially multideficient HSV vectors that can be effectively employed in several successive rounds of mutagenesis. There also exists a need for a method for efficiently inserting exogenous polynucleotides into the HSV genome, and especially a means of creating multigene HSV vectors. There exists a further need for vectors having a multiplicity of transgenes for expression in a host cell (i.e., multigene vectors), and particularly for multigene HSV vectors having independently regulated transgenes.

The present invention provides an efficient method for creating mutant HSV vectors. The present invention further provides a multigene HSV vector having independently regulated transgenes. In particular, the present invention provides an HSV vector for cancer therapy deficient for native HSV polynucleotides and containing multiple pharmacologically active therapeutic transgenes. These and other advantages of the present invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

## **BRIEF SUMMARY OF THE INVENTION**

The present invention provides a method for preparing viral vectors. The method comprises co-transfecting a source vector and a mutating cassette together into a population of appropriate host cells, such that homologous recombination occurs between the mutating cassette and the source vector whereby the mutating cassette replaces a region of the HSV genome. The mutating cassette has a unique restriction site not present in the sequence of the vector. The method further comprises plaquing the co-transfected host cells, selecting plaques in which

recombination has occurred between the source vector and the mutating cassette, and isolating the viral DNA from the plaques. The isolated viral DNA is digested with a restriction endonuclease appropriate for cleaving the viral DNA at the unique restriction site within the mutating cassette to produce two viral

5 polynucleotides. Following purification, the two viral polynucleotides can be joined to form a viral vector comprising the two viral polynucleotides.

Alternatively, the two isolated viral polynucleotides can be joined with an insertion cassette to form a viral vector comprising the insertion cassette at the former locus of the unique restriction site. The present invention further provides  
10 a mutant vector, particularly an HSV vector constructed in accordance with the method for the present invention. The present invention further provides a multigene HSV vector, particularly a multigene HSV vector for cancer therapy.

The present invention will prove highly useful in biological research. Specifically, the present invention provides reagents and methods enabling  
15 biologists to more easily study HSV molecular genetics and cytotoxicity. Additionally, the present invention provides reagents and methods permitting biologists to investigate the cell biology of viral growth and infection. Furthermore, the multigene vectors of the present invention will equip the biologist with novel tools for investigating molecular and cellular biology of gene  
20 expression and regulation in novel genetic backgrounds. Such studies, for example, can focus on the interaction between gene products in a defined or selected cellular background, the ability of transcription factors to transregulate gene expression via promoter, repressor, or enhancer elements engineered into the HSV vector, etc.

25 The present invention also will prove highly useful in the clinical setting. Specifically, the present invention permits the rapid and efficient construction of HSV vectors for gene therapy applications. Furthermore, multigene vectors of the present invention are useful vehicles for introducing batteries of therapeutic transgenes into the cells of patients to treat a host of diseases ranging from  
30 neoplasms, autoimmune diseases, neurological diseases (such as neurodegenerative disorders), hormonal imbalances, and the like.

The invention may best be understood with reference to the accompanying drawings and in the following detailed description of the preferred embodiments.

### 35 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic depiction of major steps (A-D) in the present inventive method for mutating HSV viruses.



Figures 2A-2B schematically depict pRC2, a source plasmid for the HCMV-IEp-lacZ-pA mutating cassette, as well as a method for constructing pRC2.

Figure 3 is a schematic representation of p48.2, a source plasmid for an HSV-ICP0-IEp-lacZ-pA mutating cassette.

Figure 4 is a schematic depiction of pko6, a source plasmid for HSV ICP47 homologous DNA, as well as a schematic depiction of a method for constructing pko6.

Figure 5 is a schematic depiction of pZ2, a plasmid for introducing a mutating cassette into the HSV UL41 locus, as well as a schematic depiction of a method for constructing pZ2.

Figure 6 is a schematic depiction of PB5, a plasmid for introducing a mutating cassette into the HSV ICP22 locus, as well as a schematic depiction of a method for constructing PB5 and a further step for obtaining the mutating cassette from PB5 for insertion into the pko6 plasmid.

Figure 7 is a schematic depiction of pZ1, a plasmid for introducing a mutating cassette into the HSV ICP47 locus, as well as a schematic depiction of a method for constructing pZ1 from PB5a and pko6.

Figure 8 is a schematic representation of plox-1, a source cassette for cre-lox recombination.

Figures 9A-9B are schematic representations of multideficient vectors created in accordance with the present inventive method. Figure 9A depicts examples of the products of mutation cassette insertion. Figure 9B depicts examples of the products of mutation cassette excision.

Figures 10A-10D schematically depict a method for creating multigene HSV vectors comprising non-native HSV expression cassettes in accordance with the present inventive method.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

As used herein, including the claims appended hereto, the following terms mean:

A **polynucleotide** is any portion of a nucleic acid molecule which is identified by a specific sequence of nucleotides. A polynucleotide is **expressed** if it is transcribed into an RNA transcript. A **selectable marker** is any polynucleotide whose expressed product permits a person to selectively propagate a cell that contains it. A **transgene** is a polynucleotide, the expression of which is induced or amplified in a given cellular milieu by introducing into a cell an

exogenous nucleic acid, such as a cassette containing the transgene under the control of appropriate promoters and/or transcription factors.

A **cassette** is any polynucleotide sequence having a discrete and identifiable structure. A cassette can comprise a vector or it can comprise a component of a vector. A cassette can include one or more nucleic acid sequences. An example of a cassette is one that contains a promoter element, one or more nucleic acid sequences for expression, and a polyadenylation sequence. An **expression cassette** has a polynucleotide for expression operably linked to a promoter. As used herein, when an expression cassette is identified by a particular translation product, the polynucleotide for expression of that cassette encodes the relevant translation product. Thus, for example, a "B7.1 expression cassette" comprises a polynucleotide for expression encoding B7.1 operably linked to a promoter. **Operably linked** means that separate nucleic acid sequences are functionally associated such that an event at one can precipitate a response from the other. Two or more operably linked nucleic acid sequences can, in combination, comprise an independent genetic element, such as an expression cassette. A **promoter** is a polynucleotide required for transcription at significant levels of a second polynucleotide to which it is operably linked. **IEp** refers to any immediate early promoter.

A **consensus sequence** is a DNA sequence, as well as any degenerate variation of that sequence, to which a DNA binding factor, such as a restriction endonuclease, binds with relative specific affinity. In this context, the scope of degeneracy is governed by the degree to which given nucleotides of a consensus sequence can be deleted, added, or substituted without significantly affecting the relative specificity of binding between the consensus sequence and the DNA-binding factor. A **restriction site** is a consensus sequence for a particular restriction endonuclease.

A **protein** is any molecule composed of at least two amino acids wherein each bond between the amino acids is a peptide bond, regardless of whether such molecule includes moieties other than amino acids, such as, for example, saccharides, phosphates, etc. Accordingly, the term "protein" includes polypeptides as well.

**HSV** includes any HSV-1 or HSV-2 virus or derivative thereof.

A **multigene** vector is a vector having a plurality of (i.e., two or more) non-native expression cassettes. A **multideficient** vector is a vector having a plurality of (i.e., two or more) mutations in native loci.

**Non-native** (or not native) refers to any element not present within a given system of reference. Thus, an element not native to HSV is any element not present within a wild-type HSV vector. A non-native element can comprise native sub-elements. Thus, a non-native expression cassette can comprise a native  
5 polynucleotide for expression operably linked to a non-native promoter, a non-native polyadenylation sequence, or even a native promoter, if that promoter is not operably linked to the coding polynucleotide in the native state (e.g., the operable linkage is not native).

The present invention provides a method for preparing mutant vectors. The  
10 method comprises co-transfecting a source vector and a mutating cassette together into a population of appropriate host cells such that homologous recombination occurs between the mutating cassette and the source vector to replace a portion of the gene within the homologous region (Fig. 1, step A). The mutating cassette has a unique restriction site not present in the sequence of the vector. The method  
15 further comprises plaquing the co-transfected host cells, selecting plaques in which recombination has occurred between the source vector and the mutating cassette, and isolating the viral DNA from the plaques. The isolated viral DNA is then digested with a restriction endonuclease appropriate for cleaving the viral DNA at the unique restriction site within the mutating cassette to produce two viral  
20 polynucleotides (Fig. 1, step B). The method further comprises joining (e.g., by ligation, recombination or other suitable methods) the two viral polynucleotides to form an HSV vector (Fig. 1, step C). Alternatively, the two isolated viral polynucleotides can be joined with an insertion cassette to form an HSV vector comprising the insertion cassette at the former locus of the unique restriction site  
25 (Fig. 1, step D).

The mutating cassette has a unique consensus site for a restriction endonuclease not present in the sequence of the source vector. Most preferably, the mutating cassette has two restriction sites not present in the sequence of the vector, such as two identical restriction sites. The two identical sites, thus, flank a  
30 region for excision. The region for excision flanked by the two unique restriction sites comprises any length of the cassette. For example, the restriction sites can form the borders of the nonhomologous sequence. Preferably, the region for excision flanks less than the entire nonhomologous sequence, such as, for example, a sequence encoding a selectable marker (Fig. 2, PRC2).

35 Placement of the paired unique restriction sites thus facilitates excision of the flanked region from the remainder of the viral genome. The skilled artisan will be able to engineer cassettes with such paired unique restriction sites to selectively

permit excision of some, but not all, of the cassette from the vector. Thus, for example, the mutating cassette can comprise a promoter operably linked to, and 5' of, a sequence encoding a selectable marker, which is in turn operably linked to, and 5' of, a polyadenylation sequence (see, e.g., Fig. 2). Placement of one  
5 restriction site between the promoter and the coding sequence, and the second restriction site between the coding sequence and the polyadenylation sequence, permits the selective excision of the coding sequence from the vector while retaining the promoter and the polyadenylation sequence, thereby facilitating the insertion of another coding polynucleotide.

10 The 3' and 5' regions of the mutating cassette preferably comprise polynucleotide sequences homologous to the genome of the source vector. Such homologous regions facilitate the introduction of the cassette into the source vector by homologous recombination within a suitable host cell. In this manner, a specific locus of the source vector can be targeted for mutation. Homologous  
15 recombination can target any desired loci for mutation.

The mutating cassette also can be an expression cassette, encoding any protein of interest. Preferably, the mutating cassette encodes a selectable marker for facilitating the selection of plaques comprising cells in which recombination has occurred between the source vector and the mutating cassette by screening for  
20 the selectable marker. Any known selectable marker is within the scope of the present inventive method, such as galactokinase, b-galactosidase, chloramphenicol acetyltransferase (CAT), b-lactamase, etc. A preferred selectable marker is b-galactosidase, such as the LacZ coding polynucleotide illustrated, for example, in Fig. 2. Applications in which the mutating cassette is an expression cassette, as  
25 has been described, are preferred as such applications greatly increase the efficiency of recombination mutagenesis procedures. While the expression of a selectable marker does not, by itself, overcome the inherent inefficiency of homologous recombination (as is described herein), a selectable marker permits rapid identification of even a very few recombinant plaques among many  
30 harboring unmodified source vectors.

The first step of the method for the present invention comprises co-transfecting a source vector and a mutating cassette together into a population of host cells. The present inventive method encompasses any suitable viral vector as a source vector (e.g., adenoviri, picornaviri, etc.). Preferably, the source vector is  
35 a herpesvirus vector, such as an HSV vector. A source HSV vector can be further deficient in an HSV native locus. Thus, for example, the source vector can be deficient in one or more loci such that the source vector is replication-incompetent,

such as, for example, an HSV vector deficient for the products of an essential HSV coding region (e.g., ICP4 or ICP27, see, e.g., DeLuca et al., *J. Virol.*, 56, 558-70 (1985)). In other embodiments, the source vector is replication-competent. Additionally, the source vector can have one or more non-native expression cassettes. Such non-native expression cassettes can be of any type. Preferably, a source vector has an a-tk cassette (i.e., a recombinant expression cassette comprising the HSV-tk coding polynucleotide operably linked to an immediate early promoter). For example, the ICP4 promoter promotes expression of coding polynucleotides to which it is operably linked in a strong, but transient, time course (Weir, *Proc. Nat. Acad. Sci. USA*, 90(19), 9140-44 (1993)). Employing an HSV-ICP4-IEp-tk cassette will thus enhance the safety of working with vectors produced in accordance with the present inventive method, because any unintentional recombinant within a host cell (e.g., with a wild-type HSV) possessing the a-tk allele can be rendered non-virulent (e.g., by exposure to gancyclovir).

While the present method can be employed with any viral system, when used with HSV, restriction sites generally not present in the genome include PmeI and PacI. Preferably, the unique restriction site of the expression cassette is a PacI site. Also, preferred HSV genomic targets for mutation according to the present inventive methods include, but are not limited to, the ICP4 locus, the ICP22 locus, the ICP27 locus, the ICP47 locus, the ICP0 locus, the UL41 locus, the tk locus, and the gC locus of the HSV genome. Where the target is the HSV ICP22 locus, the insertion most preferably leaves a portion of the 5' end of the coding region intact, such as, for example, from about 1 to about 300 amino acids (e.g., from about 100 to about 250 amino acids, or even about 200 amino acids), as such a mutation can reduce toxicity.

A suitable host cell is any cell in which the mutating cassette and the source vector can undergo homologous recombination to produce a vector substituting the mutating cassette for the targeted region. However, a preferred host cell is a derivative of a VERO cell. Most preferably, where the cell complements an HSV deficiency, the genome of the cell is engineered not to contain flanking regions such as, for example, N23 cells (which complement ICP27), E5 cells (which complement ICP4), and 7B cells (which complement ICP27 and ICP4). Absence of the flanking regions substantially eliminates recombination events generating a wild-type virus.

Any suitable method can be utilized to introduce polynucleotides into host cells, e.g., calcium phosphate precipitation, electroporation, liposome-mediated

transfection, microinjection, polybrene-mediated transfer, protoplast fusion, or other suitable method, and such methods are well-known in the art (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d edition, Cold Spring Harbor Press (1989); see also Watson et al., *Recombinant DNA*, Chapter 12, 2d edition, Scientific American Books (1992)).

Subsequent to cotransfer of the source vector and the mutating cassette into the host cells, the method involves plaquing the co-transfected host cells, selecting plaques in which recombination has occurred between the source vector and the mutating cassette, and isolating the viral DNA from the plaques. Methods for growing host cells to produce plaques are well-known in the art. Selecting plaques representing recombinant HSV vectors is accomplished by any means appropriate to the source vector and mutating cassette. As discussed *supra*, the mutating cassette preferably is an expression cassette encoding a selectable marker. Thus, recombinant HSV vectors are identified by plaques expressing the selectable marker. Most preferably, the selectable marker is LacZ (Fig. 2), and recombinant HSV vectors comprising the mutating cassette are identified by plaques staining blue in accordance with a standard assay for b-gal expression. Any method for subsequently isolating and purifying the recombinant viral DNA is appropriately employed within the parameters of the present inventive method. Such methods are standard procedures well-known to those skilled in the art.

The isolated recombinant viral DNA is next digested with a restriction endonuclease appropriate for cleaving the viral DNA at the unique restriction site within the mutating cassette (Fig. 1, step B). As depicted in Fig. 1, the digestion produces two viral polynucleotides, 2 and 3, each having a complementary restriction endonuclease half-site end 4 and 5, respectively. As mentioned, preferably the mutating cassette has paired unique restriction sites, such as, for example, in which the present inventive method employs two PacI sites to produce recombinant HSV vectors. It will be observed that digesting such a recombinant vector produces the polynucleotide for excision 1 between the two unique restriction sites 4 and 5, as well as the two viral polynucleotides 2 and 3. Following the digestion, the two viral polynucleotides are isolated and substantially purified.

Following substantial purification, the two viral polynucleotides can be joined to form a vector comprising the two viral polynucleotides and the unique restriction site (Fig. 1, step C). The joining can occur by any suitable method, such as by ligating the polynucleotides (blunting the ends first, if desired), by recombination between complementary regions of the polynucleotides, or other

suitable means. Moreover, the joining can occur *in vivo* or *in vitro*. The ligation and/or recombination reaction can produce several erroneous products (e.g., non-viable polynucleotides produced by juncture between two left-half or two right-half viral polynucleotides, non-viable products lacking one or the other viral polynucleotide, non-viable products in which the excised selectable marker is re-incorporated, etc.). Assaying for correct recombinant vectors, therefore, involves selecting correct plaques. Unlike the first round of the present inventive method (which involves transfecting host cells with viable virus and the mutating cassette), this second round preferably involves transfecting host cells with non-viable polynucleotides. Thus, any plaque represents a recombinant event producing a viable virus and screens for many incorrect ligation and/or recombination events producing non-viable products. Furthermore, in the preferred embodiments in which the mutating cassette has a polynucleotide encoding a selectable marker, the absence of that marker from the plaques of this second round further indicates the absence of incorrect juncture. Thus, for example, where the b-gal assay was employed following the first transfection to select for blue plaques indicative of correct insertion of the mutating cassette, white (i.e., non-staining) plaques indicate potentially correct ligation and/or recombination in the second transfection. Viral DNA isolated from plaques representing potentially correct insertions is subsequently assayed (i.e., by Southern blot analysis, sequencing, PCR, etc.) for the correct insertion product.

In some embodiments, the ligation and/or recombination reaction reconstitutes the unique restriction site from the mutating cassette, thus producing a viral vector comprising the unique restriction site 6 (Fig. 1, step C). As mentioned, in preferred embodiments the mutating cassette has two restriction sites not present in the sequence of the source vector. The result of the ligation and/or recombination reaction, thus, reconstitutes a restriction site from the two half-sites of the viral polynucleotides, producing an HSV vector comprising the unique restriction site and not comprising the region for excision from the mutating cassette. The present inventive method can be repeated through several rounds of insertion, digestion, and ligation and/or recombination to produce multideficient vectors and/or vectors comprising multiple restriction sites otherwise not present within the viral genome.

In many applications, the present inventive method is employed to engineer a vector comprising multiple unique restriction sites in one step. For example, in the first step, multiple mutating cassettes can be employed to recombine into the viral genome. Subsequent digestion and re-joining produce a viral vector

comprising deletions in each site targeted for insertion of the mutating cassette. Thus, whether in one step or several steps, the present inventive method can produce a viral vector having any number of identical or differing unique restriction sites. Such a vector permits production of multigene vectors in one  
5 subsequent step, thereby greatly increasing the efficiency in producing vectors comprising transgenes.

In addition to producing multideficient viral vectors, the present inventive method also can be employed to produce viral vectors comprising non-native expression cassettes. Preferably, as indicated in Fig. 1, the present inventive  
10 method further comprises joining the viral polynucleotides 2-3 and an insertion cassette 7 to form a vector 8 comprising the insertion cassette 7 at the former locus of the unique restriction site 1 (Fig 1, step D). While joining the polynucleotides can be carried out in any suitable fashion as described herein, the recombination occurs most efficiently within host cells which are first co-transfected with the  
15 viral polynucleotides and the insertion cassette. The molecular machinery of the host cells forms the desired vector comprising the two viral polynucleotides and the insertion cassette (Fig. 1, step D). A suitable host cell, therefore, is one in which the insertion cassette and the viral polynucleotides can be joined to produce a vector consisting essentially of the two viral polynucleotides and the insertion  
20 cassette. Lastly, the recombinant HSV vectors are plaqued, and the viral DNA is thereafter isolated and purified.

The insertion cassette can comprise any polynucleotide of interest. For example, the insertion cassette can comprise a spacer polynucleotide. In other embodiments, the insertion cassette comprises suitable functional polynucleotides,  
25 such as, for example, polynucleotides for modulating the transcription of other polynucleotides (e.g., enhancers, promoters, suppressors) or polynucleotides for expression. In preferred embodiments, the insertion cassette is an expression cassette.

Expression cassettes employed in the present inventive method are of any  
30 type appropriate for cells containing the cassette to express the protein of interest. Thus, for example, an expression cassette comprises a polynucleotide operably linked to a promoter.

Any promoter and/or enhancer sequence appropriate for controlling expression of polynucleotides from the vector can be used in constructing an  
35 expression cassette according to the present inventive method. Such promoter/enhancer elements are well known in the art. Examples of suitable promoters include prokaryotic promoters and viral promoters (e.g., retroviral ITRs,



LTRs, immediate early viral promoters, such as herpesvirus IE promoters (e.g., ICP4-IEp and ICP0-IEp), cytomegalovirus (CMV) IE promoters, and other viral promoters, such as Rous Sarcoma Virus (RSV) promoters, and Murine Leukemia Virus (MLV) promoters). Other suitable promoters are eukaryotic promoters, such as enhancers (e.g., the rabbit  $\beta$ -globin regulatory elements), constitutively active promoters (e.g., the b-actin promoter, etc.), signal specific promoters (e.g., inducible promoters, such as a promoter responsive to RU486, etc.), or tissue-specific promoters (e.g., those active in epidermal tissue, dermal tissue, tissue of the digestive organs (e.g., cells of the esophagus, stomach, intestines, colon, etc., or their related glands), smooth muscles, such as vascular smooth muscles, cardiac muscles, skeletal muscles, lung tissue, hepatocytes, lymphocytes, endothelial cells, sclerocytes, kidney cells, glandular cells (e.g., those in the thymus, ovaries, testicles, pancreas, adrenals, pituitary, etc.), tumor cells, cells in connective tissue, cells in the central nervous system (e.g., neurons, neuralgia, etc.), cells in the peripheral nervous system, or other cells of interest).

In addition to a promoter, an expression cassette for use in the present inventive method comprises a polynucleotide for expression. Preferably, the polynucleotide is a synthetic DNA, cDNA or genomic DNA fragment. Thus, the insertion cassette can encode RNA, such as biologically active antisense RNA or ribozymes, or the insertion cassette can encode biologically active proteins.

For applications in which the vector is employed to treat malignant tumors, preferred proteins are antineoplastic agents. For example, the expression cassettes can encode agents for activating prodrugs, recruiting immunoactive cells, enhancing immune response to the tumor, repressing the capacity of the tumor to destroy or suppress lymphocytes, etc. Thus, the insertion cassette can encode a-tk (such as a recombinant cassette comprising an immediate early promoter operably linked to the HSV tk coding polynucleotide), IL2, B7.1, GM-CSF, or other suitable product. Expression cassettes can comprise other polynucleotides, such as a polyadenylation sequence. Also, expression cassettes can have more than one coding polynucleotide.

For application in HSV vectors, the 5' and/or the 3' end of the insertion cassette preferably has a polynucleotide sequence complementary to the sequence within either viral polynucleotide, thus facilitating homologous pairing, as a sequence homology of about 200 bp on either side of the site of cassette insertion readily promotes recombination in HSV vectors. Of course, a homologous region can comprise a polynucleotide greater than 200 bp of homologous DNA. By facilitating homologous pairing, complementary sequences reduce the incidence of

erroneous recombination products. However, where the vector has been subject to previous rounds of mutation according to the present inventive method, homologous pairing can occur anywhere a similar cassette has been inserted, potentially decreasing the probability of correct recombination. Therefore, one of skill in the art can choose to engineer the homologous region to comprise significantly less than 200 bp. How much homology to include depends largely upon the nature of the host vector, the number of recombinant cassettes, and the degree to which the vector and/or cassettes share sequence homology. That decision is well within the ordinary skill of the art.

Preferably, juncture of the two viral polynucleotides and the insertion cassette will not reconstitute the unique restriction site. This is preferred to allow a viral vector created in accordance with the present inventive method to serve as a source vector for subsequent rounds of mutagenesis in accordance with the present inventive method.

The insertion cassette, however, can comprise a second restriction site not present within the viral genome. Such a cassette facilitates further insertion of desired cassettes at the locus by means similar to those described above. It is thus seen that cassettes can be designed to facilitate insertion of desired polynucleotide sequences at regions of interest in a single site within the viral genome as well as targeting additional sites for selective incorporation. Engineering different unique restriction sites into the mutating cassette and the insertion cassette substantially reduces the incidence of erroneous genetic manipulation in subsequent cloning steps.

The present invention further provides a mutant HSV vector. A preferred mutant HSV vector is one constructed in accordance with the method disclosed herein. HSV vectors of the present invention, most preferably, can be propagated in the absence of helper viruses (i.e., a preferred mutant HSV vector according to the present invention is a helper-free vector). Such a vector eliminates the problems attendant to most viral vector or amplicon-based systems which depend upon helper virus for adequate titers of recombinant virus, namely the generation of intolerably high frequencies of wild-type, potentially pathogenic viruses.

A mutant HSV vector in accordance with the present invention can be deficient for a native HSV locus (i.e., a polynucleotide for expression). As it is herein demonstrated that any HSV locus can be targeted for mutation, the present invention provides HSV vectors mutant in any desired locus or loci. Desirably, an HSV vector of the present invention has mutations in more than one loci (i.e., a multi-deficient HSV vector). Most preferably, the HSV vector also has a non-native HSV expression cassette.

Mutant vectors of the present invention can contain any type of mutation; suitable mutations are point mutations, temperature sensitive mutations, insertion mutations, deletion mutations, etc. Desirably, the product of a mutant HSV locus is not produced in biologically-active form, if any product is produced at all. An HSV vector according to the present invention can be deficient in any native HSV locus. Preferred mutant HSV vectors are deficient in the group of loci comprising ICP4, ICP0, tk, ICP22, ICP27, ICP47, or UL41. Removal of ICP22 expression greatly amplifies the activity of promoters not native to HSV. Furthermore, joint inactivation of the ICP4, ICP22, and ICP27 loci substantially reduces vector toxicity and reduces background expression of early and late HSV loci to virtually undetectable levels.

A mutant HSV vector according to the present invention is deficient in any desired HSV loci, and such mutant HSV vector can be a multideficient HSV vector. Of course, an HSV vector of the present invention can be deficient for three or more native HSV loci or four or more native HSV loci, such as an HSV vector deficient for ICP4, ICP22, ICP27, and UL41. The present invention also covers HSV vectors deficient for five or more native HSV loci (e.g., an HSV vector deficient for ICP4, ICP22, ICP27, UL41, and gC) or six or more native HSV loci (e.g., ICP4, ICP22, ICP27, UL41, gC, and the native HSV tk locus). In fact, HSV vectors of the present invention can be deficient in substantially any number of native HSV loci, such as vectors deficient for seven or eight HSV loci, or even substantially more HSV loci.

As mentioned, a preferred HSV vector of the present invention comprises a non-native HSV expression cassette. As the invention provides vectors with deficiencies in multiple loci, so too the vector can comprise multiple (i.e., two or more) exogenous expression cassettes (i.e., a multi-gene HSV vector). Thus, an HSV vector of the present invention can have any number of non-native expression cassettes, such as an HSV vector having three or more non-native independent expression cassettes. Preferably, an HSV vector has four or more, or even five or more non-native independent expression cassettes, and HSV vectors

of the present invention can have substantially more non-native independent expression cassettes as well (e.g., six, seven, eight or more non-native independent expression cassettes, etc.). However, the number of exogenous expression cassettes within a vector is not necessarily related to the number of deficiencies in native loci.

A non-native expression cassette can comprise a non-native promoter (i.e., a promoter not present within the wild-type HSV genome) operably linked to either a native or a non-native HSV expression polynucleotide. Similarly, a non-native expression cassette can comprise a native HSV promoter operably linked to a non-HSV coding polynucleotide, or even an HSV coding polynucleotide, if the operable linkage is not itself native to HSV. Thus an appropriate non-native expression cassette is any recombinant expression cassette having a non-native operable linkage between a promoter and a polynucleotide for expression.

A vector of the present invention can comprise any non-native HSV expression cassette. Preferably, the vector of the present invention comprises a combination of non-native cassettes and deficiencies in native loci. The identity of expression cassettes, as well as suitable combinations and permutations of expression cassettes, will depend on the purpose for which the vector is to be used. Examples of some desired promoters and coding polynucleotides are presented elsewhere herein, and it is within the skill of a researcher to select desirable cassettes for expression, alone or in combination, within a host cell.

As mentioned, multigene viruses according to the present invention are useful, in part, for clinical therapy. For example, HSV vectors for use in cancer therapy are preferably not deficient for ICP0 and contain a mutation promoting the stable expression of HSV tk, such as a mutation placing the HSV tk polynucleotide under the control of an immediate early promoter (e.g., HSV ICP4-IEp). Such a vector permits the employment of tk-based therapy. For example, tk can activate the pro-drug gancyclovir to promote increased immune surveillance and to activate the killing of tumor cells expressing tk as well as neighboring cells. Furthermore, a mutation operably linking a strong constitutive promoter to tk is preferable in vectors lacking ICP4, as otherwise tk expression diminishes (see Imbalzano et al., *J. Virol.*, 65, 565-74 (1991)). Presence of the ICP0 product strongly promotes expression from non-native HSV promoters (such as promoters in some types of non-native expression cassettes). More preferably, a vector according to the present invention further has mutations in the ICP47 locus and the UL41 locus as well. Deletion of ICP47 greatly increases immune presentation of infected cells, as the ICP47 translation normally interferes with MHC-I presentation of processed

antigen on the tumor cell (see, e.g., York et al., *Cell*, 77, 525-35 (1994)). Inactivation of the UL41 locus prevents the host shut-off mechanism from properly functioning, thus promoting full expression of foreign expression cassettes within the tumor cell.

5 For treatment of certain types of tumors, a preferred HSV vector will comprise expression cassettes conferring antitumor effects. Thus, for example, the expression cassettes can encode cytokines, enzymes for converting prodrugs (e.g., a constitutively active tk cassette), proteins increasing host immune response, or other such suitable antitumor agents. For example, suitable antitumor expression  
10 cassettes include B7.1 and B7.2, which are second-signaling molecules required for antigen presentation and which many tumor cells do not express appreciably. Other preferred antitumor expression cassettes include g-interferon, which promotes the up-regulation of MCH-I and MCH-II, which are required for antigen presentation at the tumor cell surface. Furthermore, antitumor vectors of the  
15 present invention can also comprise expression cassettes encoding cytokines, such as IL-1, IL-2, GM-CSF, etc. These molecules recruit immune T-cells and promote their proliferation and activation. Furthermore, an HSV vector of the present invention can comprise an expression cassette encoding a specific tumor antigen. Such an expression cassette greatly facilitates immune response against tumor cells  
20 expressing the cassette. Similarly, other suitable antitumor cassettes can be introduced into the vectors of the present invention.

Antitumor vectors of the present invention can be employed to combat tumors by a variety of means. For example, in some applications, a vector of the present invention is injected directly into a tumor to infect cells within the tumor.  
25 The infected cells within the tumor express the non-native expression cassettes to stimulate greater immune response against the tumor, to deliver antineoplastic agents within the tumor, to convert prodrugs into locally-effective toxins, or to effectuate some other desirable response. Additionally or alternatively, the vector of the present invention can be injected directly into a non-tumor region to infect  
30 cells *in vivo*. The infected cells express the non-native expression cassettes to stimulate greater immune response against the tumor (e.g., by expressing tumor-specific antigens), to deliver antineoplastic agents, to convert prodrugs into antineoplastic toxins, or to effectuate some other desirable response. Similarly, excised tumor cells can be infected *in vitro* and reintroduced into the region of the  
35 tumor for prophylactic as well as therapeutic effects. Moreover, isolated dendritic cells can be infected with a virus of the present invention *in vitro* and reintroduced into the patient or host. The infected dendritic cells express the non-native

expression cassettes to stimulate greater immune response against the tumor (e.g., by presenting tumor-specific antigens, by expressing immunostimulatory cytokines, etc.). These therapies can be pursued in conjunction with each other to effectively combat neoplasms within a patient.

5           A multigene HSV vector of the present invention also can be applied as a vaccine. For example, a multigene HSV vector can comprise expression cassettes encoding immunogenic epitopes. Examples of suitable expression cassettes are cassettes encoding epitopes native to viruses (e.g., coat protein epitopes of other  
10   viral epitopes), microbes (e.g., epitopes expressed by bacteria, fungi, yeast, parasites, etc.), or epitopes associated with cancers, neoplasms, cysts, or other aberrant tumors or growths. A multigene HSV vaccine vector is utilized by infecting cells of a patient with the vector to stimulate a primary immune response. The inoculation can be repeated with "booster" injections, and the patient's  
15   immuno-responsiveness can be monitored, if desired. Subsequently, an agent comprising the epitopes (e.g., a virus, microbe, tumor cell, or other agent) introduced into the inoculated patient effects a secondary response from the patient to more quickly and effectively combat the agent. When the epitopes are viral or microbial epitopes, the strategy substantially reduces or eliminates the problems attendant with the common practice of inoculation with attenuated viruses or  
20   microbes. Furthermore, the vaccine strategy can be employed in conjunction with the above-mentioned strategy of injecting tumor cells in that the secondary response against the tumor epitopes can be accentuated by infecting the tumor cells with a vector to express cytokines, immuno-recruiting agents, immuno-activating agents, and the like.

25           To further illustrate the present invention, several examples are provided below. These examples are not to be construed as in any way limiting the scope of the invention. Moreover, while this invention is described herein with an emphasis upon the foregoing preferred embodiments and the following examples, it will be obvious to those of ordinary skill in the art that variations of the preferred  
30   embodiments can be used and that it is intended that the invention can be practiced otherwise than as specifically described herein.

### EXAMPLE 1

#### STARTING MATERIAL

35           Many procedures, such as Southern blots, PCR, vector construction, including direct cloning techniques (including DNA extraction, isolation, restriction digestion, ligation, etc.), cell culture (including HAT selection and

growing cells to produce plaques), transfection of cells (including CaCl<sub>2</sub> and CaPO<sub>4</sub> transfection protocols), and b-galactosidase assays are techniques routinely performed by one of ordinary skill in the art (see generally Sambrook et al.,  
5 *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989); Graham et al., *Virol.*, 52, 456-67 (1973)).

#### Host Cells

African green monkey (Vero) cells or derivatives thereof are employed as host cells for homologous recombination and viral growth. Vero cell derivatives  
10 include E5 cells (DeLuca et al., *Nucl. Acids. Res.*, 15, 4491-4511 (1987)), which complement HSV ICP4 mutants, N23 cells, which complement for the HSV ICP27 product, and 7B cells, which complement for both ICP4 and ICP27.

N23 cells were constructed by transfecting Vero cells with a plasmid containing the HSV native ICP27 expression cassette and an expression cassette  
15 comprising the SV40 early promoter operably linked to a polynucleotide encoding neomycin phosphotransferase. Similarly, 7B cells were constructed by transfecting Vero cells with a plasmid encoding the HSV native ICP4 and ICP27 expression cassettes and an expression cassette comprising the SV40 early promoter operably linked to a polynucleotide encoding neomycin  
20 phosphotransferase

#### Viruses

##### d120

The d120 virus has been previously described (DeLuca et al., *J. Virol.*, 56, 558-70 (1985)). This virus was used as a source vector for constructing the  
25 multideficient HSV vectors described herein.

Briefly, this virus is deficient for both copies of the native HSV ICP4 locus. Thus, the d120 virus can only replicate in cells which complement ICP4 (e.g., E5 cells).

##### 5dl1.2

30 The 5dl1.2 virus has been previously described (McCarthy et al., *J. Virol.*, 63, 18-27 (1989)). This virus was used as a source vector for constructing the multideficient HSV vectors described herein.

Briefly, this virus is deficient for the native HSV ICP27 locus. Phenotypically, the 5dl1.2 virus can only replicate in cells which complement  
35 ICP27 (e.g., N23 cells).

## DSma-UL41

The DSma Virus has been previously described (Read et al., *J. Virol.*, 67, 7149-60 (1993)). This virus is deficient for the native HSV UL41 locus. This vector was used as a source vector for constructing the multideficient HSV vectors described herein.

## Ktk-lox

This HSV vector is deficient for tk (i.e., tk<sup>-</sup>) by virtue of the insertion of a lox-recognition site within the polynucleotide encoding tk.

## Plasmids

## 10 pko6

This plasmid was constructed to introduce a mutation into the ICP47 locus by homologous recombination, and pko6 is depicted in Fig. 4. When recombined into the HSV genome, a roughly 1.1 kb polynucleotide within the ICP47 locus from the ApaLI site (145443) to the NcoI site (144332) is deleted. Furthermore, between these two restriction sites, ko6 is engineered to contain PstI and XbaI sites for insertion of a desired cassette (such as a mutating cassette of the present inventive method).

To construct ko6, the EcoRI-EcoRI "H" fragment was digested with BglII and KpnI to isolate a roughly 2.1 kb polynucleotide (124758-144892), which was ligated into the polylinker of pSP72 (BglII, KpnI). This plasmid was termed 47-left-flank. Subsequently, the 47-left-flank was digested with KpnI and NcoI to remove the fragment spanning from 144332-144892 of the HSV genome, thereby removing the coding sequences for US10 and US11 while leaving the polyadenylation site in tact. The ends were then blunted and the plasmid religated to form pBD2.

Similarly, the EcoRI-EcoRI "H" fragment was digested with EcoRI and KpnI to isolate a roughly 1.8 kb polynucleotide (144892-146693), which was ligated into the polylinker of pSP72 (EcoRI, KpnI). This plasmid was termed 47-right-flank. Subsequently, the 47-right-flank was digested with ApaLI, the ends blunted, and the plasmid religated with PstI linkers. A PstI digestion, thus, removed the polynucleotide between the EcoRI and ApaLI of the ICP47 locus, which was ligated into the pBD2 plasmid (digested with PstI) to form ko6.

Finally, ko6 was digested with XhoI and EcoRI and religated with PmeI linkers to remove the XhoI and EcoRI sites from the plasmid. The resultant plasmid is pko6.

When homologously recombined into the HSV genome, ko6 deletes US10, US11, and ICP47 while leaving the polyadenylation site in tact. Furthermore, the



internal XbaI and PstI sites facilitate introduction of a cassette within the region of this deletion.

#### PRC2

5 This plasmid contains a HCMV-IEp-lacZ-SV40pA mutating cassette for insertion into the HSV native ICP47 or ICP22 locus. The cassette is located within a XbaI-PstI fragment on the plasmid and contains two PacI sites. Furthermore, PB5 is engineered to introduce a mutation into the ICP22 locus by homologous recombination. The construction of PB5 is indicated in Fig. 6.

10 The PacI-LacZ cassette was cloned into this plasmid as follows. The PIEP-LacZ source plasmid contains a HCMV-IEp-LacZ cassette within the pBR322 plasmid. A PacI site was introduced into the cassette by mutating the HindIII site by blunting it using the Klenow fragment of DNA polymerase I, and then ligating a PacI linker to the blunt end, followed by religation of the plasmid to create LP4 (Fig. 2A, step A). Similarly, a PacI site was introduced into the EcoRV  
15 site within the pBluescript<sup>®</sup> II SK<sup>-</sup> plasmid to create pBSSK-PacI (Fig. 2A, Step B).

Following digestion, a BglII-PstI polynucleotide from LP4 was ligated into the BamHI-PstI site of pBSSK-PacI to create the LPX plasmid (Fig. 2A, steps C & D).

20 The XbaI sites (both within the cassette 5' of the novel PacI site and within the polylinker pBSSK-II polylinker) were changed to BglII, and the HindIII site was changed to an XbaI site, to create the plasmid AXLX (Fig. 2B, step E). Furthermore, the HindIII site within the pRc-CMV<sup>™</sup> vector was replaced with a ClaI site, and the BbsI site following the BGHpA polynucleotide was replaced  
25 with a HindIII site, to create pRC2 (Fig. 2B, step F).

Subsequently, the BglII-XbaI polynucleotide comprising the LacZ-Sv40pA sequences from AXLX was ligated into the BamHI-XbaI site of pRC2 to create the PRC2 plasmid (Fig. 2B, steps G-I).

#### PB5

30 This plasmid (Fig. 6) facilitates homologous recombination into the ICP22 locus.

The BamHI-BamHI "N" fragment was digested with BamHI and KpnI to isolate a roughly 3.4 kb polynucleotide (131398-134787), which was ligated into the polylinker of pSP72 (BamHI, KpnI) to create the bamN plasmid.

35 Subsequently, this plasmid was digested with PstI (cutting at 133372), the ends blunted, and the plasmid relegated with SalI linkers to create pBSSK-bam/kpn of bamN(pst)-salI.

The pBSSK-bam/kpn of bamN(past)-sall plasmid, thus, contains homologous regions to ICP22. Insertion into the ICP22 locus causes a mutation in which the ICP22 protein is truncated at roughly 200 amino acids. By virtue of the PstI site, an exogenous polynucleotide can be cloned into pBSSK-bam/kpn of  
5 bamN(past)-sall as an HindIII-Sall polynucleotide.

The Sall-HindIII polynucleotide comprising the LacZ-SV40pA sequences from the PRC2 plasmid (Fig. 2) was ligated into the Sall-HindIII site of pBSSK-bam/kpn of bamN(past)-sall to create PB5, following the appropriate restriction digestion.

10 The LacZ-SV40pA polynucleotide can be removed from PB5 by digesting the plasmid with ClaI and XbaI, which cut outside the double PacI-flanked polynucleotide and between the Sv40pA sequence and the BGHpA sequence (Fig. 2). Thus, an exogenous polynucleotide can be inserted as a ClaI-XbaI polynucleotide, operably linked to both the HCMV-IEp and the remaining  
15 BGHpA.

For insertion into the ICP47 locus, the PstI site in the SV40pA of PB5 is destroyed, and the Sall site in the polylinker changed to a PstI site for excision as a PstI-XbaI fragment (pB5A, Fig. 6). Thus, the mutating cassette within PB5 can be cloned into the XbaI-PstI sites of ko6.

20 UL41 mutation (p48.2)

As indicated in Fig. 5, a 6.6 kb EcoRI-HindIII polynucleotide comprising the entire UL41 locus was cloned into the polylinker of pBSSK. The plasmid was thereafter digested with NotI (which cuts at several sites within the region) to retain a 3.7 kb NotI-HindIII polynucleotide (90145-93858 of the HSV genome)  
25 within the plasmid. Subsequently, this plasmid was digested with SmaI (which cuts twice within the NotI-HindIII polynucleotide) to excise a 583 SmaI-SmaI polynucleotide representing portions of exon 3 of the UL41 coding region.

A plasmid (p48.2) (Fig. 3) was created to contain the ICP0-IEp-LacZ-SV40pA mutating cassette for insertion into the HSV UL41 locus. A  
30 polynucleotide comprising the HSV-1 ICP0-SV40IEp was cloned into the EcoRV site of the plasmid such that it was operably linked to the b-gal construct. The cassette is located within a XhoI-NotI polynucleotide on the plasmid and contains two PacI sites flanking the cassette.

p48.2 was digested with XhoI-NotI to isolate the polynucleotide  
35 comprising the mutating cassette. This polynucleotide was then blunted and cloned into the SmaI linearized plasmid to create pZ2 (Fig. 5) PZ2 facilitates

homologous recombination of the HSV-ICP0-LacZ-SV40pA expression cassette into the ICP22 locus.

#### gC Insertion

5       The pGC/HCMVg5-GAL4:VP16 vector (Oligino et al., *Gene Therapy*, 3, 892-99 (1996)) was digested to replace the GAL4:VP16 cassette with a mutating cassette comprising two PacI sites flanking the lacZ coding polynucleotide to create the pGC-lac plasmid. This plasmid, thus, creates a cassette in which the lacZ coding polynucleotide is under the regulation of the HCMV-IEp and is  
10       flanked by sequence homologous to the HSV gC locus.

#### Source of cassettes for cre-lox recombination into the tk locus

      A plasmid (pLox-I) (Fig. 8) was constructed comprising two expression cassettes and a lox site for site-specific recombination. This plasmid has a BamHI-BstEII fragment comprising an HSV expression cassette in which the HSV  
15       tk coding polynucleotide is operably linked to the HSV-ICP4 IEp. The plasmid also has a BamHI-BamHI polynucleotide comprising an expression cassette into which the HCMV-IEp is operably linked to a b-galactosidase coding polynucleotide. The lox site is placed between the two cassettes.

20       A second plasmid (pLox-II) similar to pLox-I was also created, but which lacks the HCMV-IEp-b-gal cassette.

#### Source of ICP4-IEp-tk (a-tk)

      A cassette comprising the HSV ICP4-IEp operably linked to the coding region for the HSV-tk coding polynucleotide was created by ligating the "BamP" BamHI-BamHI fragment spanning the UL22-24 loci of HSV1 into the BamHI  
25       site of pUC-19 to create the pUCX1 plasmid. The plasmid was then digested with BglII and PstI to excise a fragment representing the native HSV tk promoter and a portion of the UL24 locus. A BglII-HindIII polynucleotide comprising the HSV ICP4-IEp was ligated to the BglII end of the linearized plasmid such that the promoter was operably linked to the tk coding polynucleotide. Subsequently, the  
30       plasmid was reconstructed to create an HSV ICP4-IEp-tk cassette within the BamHI-BamHI polynucleotide.

#### Source of HCMV-IEp-hIL-2

      A cDNA encoding human IL-2 (ATCC 39673 Genetics Institute) was ligated to the 3' untranslated region of the rabbit b-globin gene and the SV40pA  
35       sequence, further ligated to place the HCMV-IEp and b-globin intron 5' of the IL-2 coding sequence, and cloned into the pko6 to replace the HCMV-lacZ cassette in pko6.

## Source of HCMV-IEp-hB7.1

A cDNA encoding human B7.1 was cloned into the pB5 plasmid such that the coding nucleotide was placed under the control of the HCMV-IE promoter.

## Source of ICP0-IEp-hGM-CSF

- 5 A cDNA encoding human hGM-CSF (ATCC 57594) was end-modified to form an EcoRI-XbaI fragment and ligated into p41 to replace the lacZ coding sequence and place the coding sequence under control of the HSV ICP0 promoter.

**EXAMPLE 2**10 **CREATION OF FIRST ROUND MUTANTS**

In order to investigate the efficacy of the present inventive method to create singly and multiply-deficient HSV viruses, a number of mutant viruses were created. Details regarding the construction of each vector are presented in the following subsections.

- 15 Each of the viruses was assayed for growth characteristics on 7B cells, E5 cells, N23 cells, and Vero cells, and the genotypes were analyzed through Southern blotting for native tk, ICP22, UL24, UL41, and ICP47. Furthermore, the titer of each stock was measured. These data are presented in Table 1.

20 Table 1  
Genotype and Titer for Deficient HSV Vectors

Vector	Growth Characteristics				Non-Essential Genes Status					(10 <sup>6</sup> )/10 <sup>6</sup> cells
	7B	E5	N23	Vero	TK	ICP22	UL24	UL41	ICP47	
SHZ.1	+	+	-	-	-	+	+	+	+	NT
SHZ.2	+	+	-	-	*	+	+	+	+	20000
SOZ.1	+	+	-	-	*	+	-	-	+	2500
S4TK	+	+	-	-	*	+	-	+	+	2777
DHZ.1	+	+	-	-	*	-	-	+	+	180
DHZ.2	+	+	-	-	*	+	-	+	-	1782
DHZ.3	+	-	+	-	+	-	+	+	+	210
THZ.1	+	-	-	-	*	-	-	+	+	114
THZ.3	+	-	-	-	*	-	-	-	+	408
TOZ.1	+	-	-	-	*	-	-	-	+	371
D.1	+	+	-	-	*	-	-	+	+	ND
D.2	+	+	-	-	*	+	-	+	-	ND
T.1	+	-	-	-	*	-	-	+	+	ND
T.3	+	-	-	-	*	-	-	-	+	ND

ND= No Data

## SHZ.1

- 25 This vector is tk<sup>-</sup>, ICP4<sup>-</sup>, and contains an HCMV-IEp-LacZ expression cassette at the native tk locus. SHZ.1 is represented in Fig. 9A. This vector was

created to assay the ability to insert an exogenous polynucleotide at the lox cite in the Ktk-lox Vector.

SHZ.1 was created by cre-lox-mediated recombination between the d120 tk-lox HSV vector and the plox-II plasmid. Following the recombination reaction, the viruses were precipitated into E5 cells and assayed for blue plaques by the b-gal assay. Blue plaques were isolated and the correct insertion was confirmed by Southern blot hybridization.

Growth characteristics and genotype are presented in Table 1.

#### SHZ.2

This vector is ICP4<sup>-</sup> and contains both an HCMV-LacZ expression cassette and an HSV-ICP4-IEp-tk expression cassette at the tk locus. SHZ.1 is represented in Fig. 9A.

SHZ.2 was created by cre-lox-mediated recombination between the d120 tk-lox HSV vector and the plox-I plasmid. Following the recombination reaction, the viruses were precipitated into E5 cells and assayed for blue plaques by the b-gal assay and for the presence of tk expression. Blue plaques were isolated, and the correct insertion was confirmed by Southern blot hybridization.

Growth characteristics and genotype are presented in Table 1.

#### S4TK

This vector is ICP4<sup>-</sup>, and contains an ICP4-IEp-tk expression cassette at the UL24 locus. S4tk is depicted schematically in Fig. 10A, step A.

This vector was created in two steps (Fig. 10A, step A). First, the BamHI-BamHI polynucleotide comprising the HSV-ICP4-IEp-tk cassette from ploxI was introduced into the HSV genome by homologous recombination into the tk locus of the Ktk-lox vector. Desired recombinants would rescue the tk<sup>+</sup> phenotype by eliminating the lox recognition sequence present in Ktk-lox and by placing the tk coding polynucleotide under the control of the constitutive HSV ICP4-IEp; furthermore, the desired recombinants would lack UL24 by virtue of the placement of the exogenous ICP4 promoter within the UL24 coding polynucleotide. Thus, recombinants were selected by the HAT assay in tk<sup>-</sup> cells. A desired recombinant virus was selected, and the correct insertion confirmed by Southern hybridization.

The recombinant, termed ICP4TK KOS was crossed with the d120 vector via homologous recombination to produce a vector deficient for ICP4 and which had the HSV ICP4-IEp-tk cassette. Recombinants were screened for viruses that could form semi-syncytial plaques on E5 cells but not on VERO cells. One virus (S4TK, Fig. 10A, step A) was purified, and the genotype confirmed by Southern hybridization.

Growth characteristics and genotype are presented in Table 1.

#### SOZ.1

This vector is ICP4<sup>-</sup>, and UL41<sup>-</sup> and contains an HSV-ICP4-IEp-tk expression cassette at the UL24 locus, and an HSV-ICP0-IEp-LacZ mutating  
5 cassette the UL41 locus. SOZ.1 is depicted in Fig. 9A and Fig. 10C, step F.

This vector was created by homologous recombination to insert the ICP0-IEp-LacZ mutating cassette from the pZ2 plasmid into the HSV UL41 locus of S4TK. Recombinants were screened by X-gal staining, and viral DNA from blue  
10 plaques was purified and subjected to Southern blot analysis to confirm the correct insertion.

Growth characteristics and genotype are presented in Table 1.

#### DHZ.1

This vector is ICP4<sup>-</sup> and ICP22<sup>-</sup>, and it contains an HSV-ICP4-IEp-tk expression cassette at the UL24 locus and an HCMV-IEp-lacZ-pA mutating  
15 cassette at the ICP22 locus. DHZ.1 is depicted in Fig. 9A.

To create the DHZ.1 vector, a KpnI-NotI fragment from PB5 comprising the ICP22 homologous regions and the HCMV-IEp-lacZ-pA mutating cassette was co-transfected into E5 cells with the S4TK vector. Recombinants were screened for LacZ expression, and the correct insertion was confirmed with Southern blot  
20 analysis.

Growth characteristics and genotype are presented in Table 1.

Subsequently, the D.1 vector was created by PacI excision. DHZ.1 DNA was digested with PacI to excise the lacZ expression cassette from the ICP22 locus. Viral DNA fragments were then transfected, and the resultant plaques were  
25 assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were selected and assayed for correct ligation by Southern analysis. A plaque with the correct excision (D.1, Fig. 9B) was amplified. D.1, thus, is ICP4<sup>-</sup> and ICP22<sup>-</sup> (by virtue of the excision of the PacI-flanked cassette), and it contains an ICP4-IEp-tk expression cassette at the UL24 locus but lacks LacZ.

#### DHZ.2

This vector is ICP4<sup>-</sup> and ICP47<sup>-</sup>, and it contains an HSV-ICP4-IEp-tk expression cassette at the UL24 locus. DHZ.2 is depicted in Fig. 9A.

To create the DHZ.2 vector, a BglII-PmeI fragment from pko6 comprising the ICP47 homologous regions flanking the HCMV-IEp-pA cassette was co-  
35 transfected into E5 cells with the S4TK vector (Fig 10A, Step B). Recombinants

were screened for LacZ expression, and the correct insertion was confirmed with Southern blot analysis.

Growth characteristics and genotype are presented in Table 1.

Subsequently, the D.2 vector was created by PacI excision. DHZ.2 DNA  
5 was digested with PacI to excise the lacZ expression cassette from the ICP47  
locus. Viral DNA fragments were then transfected, and the resultant plaques were  
assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were  
selected and assayed for correct ligation by Southern blot analysis. A plaque with  
the correct excision (D.2, Fig. 9B) was amplified. D.1, thus, is ICP4<sup>-</sup> and ICP47<sup>-</sup>  
10 (by virtue of the excision of the PacI-flanked cassette), and it contains an ICP4-  
IEp-tk expression cassette at the UL24 locus but lacks LacZ.  
DHZ.3

This vector is tk<sup>+</sup>, ICP22<sup>-</sup>, and ICP27<sup>-</sup> and contains the HCMV-IEp-lacZ-  
pA mutating cassette at the ICP22 locus. DHZ.3 is depicted in Fig. 9A.

15 To create the DHZ.3 vector, a KpnI-NotI fragment from PB5 comprising  
the ICP22 homologous regions and the HCMV-LacZ-IEp-pA mutating cassette  
was co-transfected into N23 cells with the 5dl1.2 vector. Recombinants were  
screened for LacZ expression, and the correct insertion was confirmed with  
Southern blot analysis.

20 Growth characteristics and genotype are presented in Table 1.

THZ.1

This vector is ICP4<sup>-</sup>, ICP22<sup>-</sup>, and ICP27<sup>-</sup>, and it contains an ICP4-IEp-tk  
expression cassette at the UL24 locus and an HCMV-IEp-lacZ-pA mutating  
cassette at the ICP22 locus. THZ.1 is depicted in Fig. 9A.

25 This vector was created by homologous recombination between the DHZ.1  
vector and the 5dl1.2 vector in 7B cells. Recombinants were screened by X-gal  
staining, and viral DNA from blue plaques was purified and subjected to Southern  
blot analysis to confirm the correct genotype.

Growth characteristics and genotype are presented in Table 1.

30 Subsequently, the T.1 vector was created by PacI excision. THZ.1 DNA  
was digested with PacI to excise the lacZ expression cassette from the ICP22  
locus. Viral DNA fragments were then transfected, and the resultant plaques were  
assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were  
selected and assayed for correct ligation by Southern blot analysis. A plaque with  
35 the correct excision (T.1, Fig. 9B) was amplified. T.1, thus, is ICP4<sup>-</sup>, ICP22<sup>-</sup>, and  
ICP27<sup>-</sup> (by virtue of the excision of the PacI-flanked cassette), and it contains an  
ICP4-IEp-tk expression cassette at the UL24 locus but lacks LacZ.

## THZ.3

This vector is ICP4<sup>-</sup>, ICP22<sup>-</sup>, ICP27<sup>-</sup>, and UL41<sup>-</sup>, and it contains an ICP4-IEp-tk expression cassette at the UL24 locus and the HCMV-IEp-lacZ-pA mutating cassette at the ICP22 locus. THZ.3 is depicted in Fig. 9A.

5 This vector was created by homologous recombination between the THZ.1 vector and the DSma-UL41 vector in 7B cells. Recombinants were screened by X-gal staining, and viral DNA from blue plaques was purified and subjected to Southern blot analysis to confirm the correct genotype.

Growth characteristics and genotype are presented in Table 1.

10 Subsequently, the T.3 vector was created by PacI excision. THZ.3 DNA was digested with PacI to excise the lacZ expression cassette from the ICP22 locus. Viral DNA fragments were then transfected, and the resultant plaques were assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were selected and assayed for correct ligation by Southern analysis. A plaque with the  
15 correct excision (T.3, Fig. 9B) was amplified. T.3, thus, is ICP4<sup>-</sup>, ICP22<sup>-</sup> (by virtue of the excision of the PacI-flanked cassette), ICP27<sup>-</sup>, and UL41<sup>-</sup>, and it contains an ICP4-IEp-tk expression cassette at the UL24 locus but lacks LacZ.  
TOZ.1

20 This vector is ICP4<sup>-</sup>, ICP22<sup>-</sup>, ICP27<sup>-</sup>, and UL41<sup>-</sup>, and it contains an ICP4-IEp-tk expression cassette at the UL24 locus.

T.1 DNA and SOZ.1 DNA were co-transfected into 7B cells to promote homologous recombination. Resultant plaques were assayed for b-galactosidase activity and blue plaques further assayed for the ability to grow on E5 or N23 cells. The correct recombination was confirmed with Southern Analysis.

25 Growth characteristics and genotype are presented in Table 1.

## Discussion

This example has demonstrated that novel HSV strains deficient for a multiplicity of native loci can readily be created using the insertion of a mutating cassette such as those described herein. Furthermore, this example evinces the  
30 power of the present inventive method at creating novel mutations in discrete loci by excising the double PacI cassette.

**EXAMPLE 3****CREATION OF INSERTION MUTANTS**

35 To investigate the efficiency of mutagenesis by the PACI recombination method, a variety of insertion mutants were constructed. Details concerning the creation of each vector are presented in the following subsections.



Efficiency was tested by noting the percentage of white plaques in the blue background (indicating any recombinant event) and by noting the percentage of correct insertions among white plaques (indicating the efficiency of correct insertion). The results are presented in Table 2.

Table 2

Vector	Source	Insert	% White	Recombination
3A3	DHZ.1	HCMV-hB7 in ICP22	88%	20%
HvIL-10	THZ.1	SCMV-vIL-10 in ICP22	88%	58%
HG1	SOZ.1	ICP0-hGM-CSF in UL41	100%	58%
T/Hbcl2	THZ.1	HCMV-bcl-2 in ICP22	100%	50%
DHIL2.2/ 167.1	DHZ.2	HCMV-hIL-2 in ICP47	100%	65%
6.8.1	THZ.3	HCMV-hB7.1 in ICP47	60%	66%
PM15	THZ.3	HCMV-mB7.1 in ICP22	100%	30%
MG2	SOZ.1	ICP0-mGM-CSF in UL41	80%	19%
M7	THZ.1	HCMV-muc1 in ICP22	100%	15%

### 3A3

This vector is ICP4<sup>-</sup> and ICP22<sup>-</sup>, contains an HSV-ICP4-IEp-tk expression cassette at the UL24 locus, and contains an HCMV-IEp-hB7 expression cassette at the ICP22 locus.

DHZ.1 vector DNA was digested with PacI, and the viral DNA was purified. A linear polynucleotide comprising an HCMV-IEp-hB7 expression cassette flanked by regions homologous to ICP22 was co-transfected with the DNA arms into 7B cells to promote recombination. The resultant plaques were assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were selected and assayed for correct recombination of the HCMV-IEp-hB7 expression cassette into the ICP22 locus by Southern blot analysis. The efficiency of this recombination is presented in Table 2.

### HvIL-10

This vector is ICP4<sup>-</sup>, ICP22<sup>-</sup>, and ICP27<sup>-</sup>; it contains an ICP4-IEp-tk expression cassette at the UL24 locus and an HCMV-IEp-vIL10 expression cassette at the ICP22 locus.

THZ.1 vector DNA was digested with PacI, and the viral DNA was purified. A linear polynucleotide comprising an HCMV-IEp-vIL10 expression cassette flanked by regions homologous to ICP22 was co-transfected with the viral DNA arms into 7B cells to promote recombination. The resultant plaques were assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were selected and assayed for correct recombination of the HCMV-IEp-vIL10

expression cassette into the ICP22 locus by Southern analysis. The efficiency of this recombination is presented in Table 2.

#### HG1

5 This vector is ICP4<sup>-</sup> and UL41<sup>-</sup>, and it contains an ICP4-IEp-tk expression cassette at the UL24 locus and an HSV-ICP0-hGM-CSF expression cassette at the UL41 locus (Fig. 10C, step F).

SOZ.1 vector DNA was digested with PacI, and the viral DNA was purified. A linear polynucleotide comprising an HSV-ICP0-hGM-CSF expression cassette flanked by regions homologous to UL41 was co-transfected with the viral  
10 DNA arms into 7B cells to promote recombination. The resultant plaques were assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were selected and assayed for correct recombination of the HSV-ICP0-hGM-CSF expression cassette into the UL41 locus by Southern blot analysis. The efficiency of this recombination is presented in Table 2.

#### 15 T/Hbcl2

This vector is ICP4<sup>-</sup>, ICP22<sup>-</sup>, and ICP27<sup>-</sup>; it contains an ICP4-IEp-tk expression cassette at the UL24 locus and an HCMV-IEp-bcl2 expression cassette at the ICP22 locus.

THZ.1 vector DNA was digested with PacI, and the viral DNA was  
20 purified. A linear polynucleotide comprising an HCMV-IEp-bcl2 expression cassette flanked by regions homologous to ICP22 was co-transfected with the viral DNA arms into 7B cells to promote recombination. The resultant plaques were assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were selected and assayed for correct recombination of the HCMV-IEp-bcl2 expression  
25 cassette into the ICP22 locus by Southern blot analysis. The efficiency of this recombination is presented in Table 2.

#### DHIL2.2/167.1

This vector is ICP4<sup>-</sup> and ICP47<sup>-</sup>, and it contains an HCMV-LacZ expression cassette at the UL24 locus and an HCMV-IL2 expression cassette at the  
30 ICP47 locus (Fig. 10, steps C-D).

DHZ.2 vector DNA was digested with PacI, and the viral DNA was purified. A linear polynucleotide comprising an HCMV-IEp-bcl2 expression cassette flanked by regions homologous to ICP47 was co-transfected with the viral  
35 DNA arms into 7B cells to promote recombination. The resultant plaques were assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were selected and assayed for correct recombination of the HCMV-IEp-bcl2 expression

cassette into the ICP47 locus by Southern blot analysis. The efficiency of this recombination is presented in Table 2.

#### 6.8.1

5 This vector is ICP4<sup>-</sup>, ICP22<sup>-</sup>, ICP27<sup>-</sup>, and UL41<sup>-</sup>; it contains an ICP4-IEp-tk expression cassette at the UL24 locus and an HCMV-IEp-hB7.1 expression cassette at the ICP22 locus.

10 THZ.3 vector DNA was digested with PacI, and the viral DNA was purified. A linear polynucleotide comprising an HCMV-IEp-hB7.1 expression cassette flanked by regions homologous to ICP22 was co-transfected with the viral DNA arms into 7B cells to promote recombination. The resultant plaques were assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were selected and assayed for correct recombination of the HCMV-IEp-hB7.1 expression cassette into the ICP22 locus by Southern blot analysis. The efficiency of this recombination is presented in Table 2.

#### 15 PM15

This vector is ICP4<sup>-</sup>, ICP22<sup>-</sup>, ICP27<sup>-</sup>, and UL41<sup>-</sup>; it contains an ICP4-IEp-tk expression cassette at the UL24 locus and an HCMV-IEp-mB7.1 expression cassette at the ICP22 locus.

20 THZ.3 vector DNA was digested with PacI, and the viral DNA was purified. A linear polynucleotide comprising an HCMV-IEp-mB7.1 expression cassette flanked by regions homologous to ICP22 was co-transfected with the viral DNA arms into 7B cells to promote recombination. The resultant plaques were assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were selected and assayed for correct recombination of the HCMV-IEp-mB7.1 expression cassette into the ICP22 locus by Southern blot analysis. The efficiency of this recombination is presented in Table 2.

#### MG2

30 This vector is ICP4<sup>-</sup> and UL41<sup>-</sup>, and it contains an ICP4-IEp-tk expression cassette at the UL24 locus and an HSV-ICP0-mGM-CSF expression cassette at the UL41 locus.

35 SOZ.1 vector DNA was digested with PacI, and the viral DNA was purified. A linear polynucleotide comprising an HSV-ICP0-mGM-CSF expression cassette flanked by regions homologous to UL41 was co-transfected with the viral DNA arms into 7B cells to promote recombination. The resultant plaques were assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were selected and assayed for correct recombination of the HSV-ICP0-

mGM-CSF expression cassette into the UL41 locus by Southern blot analysis. The efficiency of this recombination is presented in Table 2.

M7

This vector is ICP4<sup>-</sup>, ICP22<sup>-</sup>, ICP27<sup>-</sup>, and UL41<sup>-</sup>; it contains an ICP4-IEp-tk expression cassette at the UL24 locus and an HCMV-IEp-muc1 expression cassette at the ICP22 locus.

THZ.3 vector DNA was digested with Pac1, and the viral DNA was purified. A linear polynucleotide comprising an HCMV-IEp-muc1-pA expression cassette not flanked by regions homologous to ICP22 (but flanked by regions homologous to the HCMV and pA sequences of the insertion cassette) was co-transfected with the viral DNA arms into 7B cells to promote recombination. The resultant plaques were assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were selected and assayed for correct recombination of the HCMV-IEp-muc1 expression cassette into the ICP22 locus by Southern blot analysis. The efficiency of this recombination is presented in Table 2.

#### Discussion

This example details the creation of nine separate insertion mutants via the present inventive method. Moreover, the data presented in Table 2 indicate that the present inventive method for introducing a foreign expression cassette into a targeted position within the HSV genome results in the correct insertion approximately 38% of the time. Lastly, the data indicate that viral flanking regions are not necessary to produce desired recombinants. Thus, the Pac1 recombination method for creating mutant HSV viruses is significantly more efficient than traditional methods for creating predefined HSV deletions/insertions.

25

### EXAMPLE 4

#### CREATION OF MULTIGENE HSV VECTORS

To examine the feasibility of performing successive rounds of Pac1 mutagenesis, a series of successive rounds was conducted, in combination with homologous recombination, to develop an HSV vector in which 4 exogenous expression cassettes operate in a background lacking four native HSV loci. This mutagenesis scheme is graphically represented in Fig. 10A-10C.

7G6

This vector is ICP4<sup>-</sup>, ICP22<sup>-</sup>, and ICP47<sup>-</sup>, and it contains an ICP4-IEp-tk expression cassette at the UL24 locus and an HCMV-IL2 expression cassette at the ICP47 locus (Fig. 10B, step D).

35

To create the 7G6 vector, a KpnI-NotI fragment from PB5 comprising the ICP22 homologous regions and the HCMV-IEp-LacZ-pA cassette from pRC2 was co-transfected into 7B cells with the DHIL2.2/167.1 vector (Fig. 10B, step D). Recombinants were screened for LacZ expression, and the correct insertion was confirmed with Southern blot analysis.

#### 7H10

This vector is ICP4<sup>-</sup>, ICP22<sup>-</sup>, and ICP47<sup>-</sup>, and it contains an ICP4-IEp-tk expression cassette at the UL24 locus, an HCMV-IL2 expression cassette at the ICP47 locus, and an HCMV-IEp-hB7.1 expression cassette at the ICP22 locus (Fig. 10B, step E).

To create the 7H10 vector, 7G6 vector DNA was digested with PacI, and the viral DNA was purified. A linear polynucleotide comprising an HCMV-IEp-hB7.1 expression cassette flanked by regions homologous to ICP22 was co-transfected with the viral DNA arms into 7B cells to promote recombination (Fig. 10B, step E). The resultant plaques were assayed for b-galactosidase activity. LacZ negative (i.e., white) plaques were selected and assayed for correct recombination of the HCMV-IEp-hB7.1 expression cassette into the ICP22 locus by Southern blot analysis.

#### HGZ.25

This vector is ICP4<sup>-</sup>, ICP27<sup>-</sup>, ICP22<sup>-</sup>, and UL41<sup>-</sup>, and it contains an HSV-ICP4-IEp-tk expression cassette at the UL24 locus, an HSV-ICP0-hGM-CSF expression cassette at the UL41 locus, and an HCMV-IEp-lacZ-pA mutating cassette at the ICP22 locus (Fig. 10D, Step G).

HG.1 and THZ.1 were co-infected into 7B cells to promote homologous recombination. Resultant plaques were assayed for b-galactosidase activity and blue plaques further assayed for the inability to grow on E5 or N23 cells. The correct recombination was confirmed with Southern blot analysis.

#### HX86

This vector is ICP4<sup>-</sup>, ICP27<sup>-</sup>, ICP22<sup>-</sup>, ICP47<sup>-</sup>, and UL41<sup>-</sup>; it contains an HSV-ICP4-IEp-tk expression cassette at the UL24 locus, an HSV-ICP0-hGM-CSF expression cassette at the UL41 locus, a HCMV-IEp-hB7.1 expression cassette at the ICP22 locus, and a HCMV-IEp-hIL2 expression cassette at the ICP47 locus (Fig. 10D, step H).

7H10 and HGZ.25 were co-infected into 7B cells to promote homologous recombination to result in a crossover event between the UL41 locus and the ICP22 locus. Resultant plaques were assayed for b-galactosidase activity, and

white plaques were further assayed for the inability to grow on E5 or N23 cells. The correct recombination was confirmed with Southern blot analysis.

#### HX86Z

5 This vector is ICP4<sup>-</sup>, ICP27<sup>-</sup>, ICP22<sup>-</sup>, ICP47<sup>-</sup>, gC<sup>-</sup> and UL41<sup>-</sup>; it contains an HSV-ICP4-IEp-tk expression cassette at the UL24 locus, an HSV-ICP0-Iep-hGM-CSF expression cassette at the UL41 locus, an HCMV-IEp-hB7.1 expression cassette at the ICP22 locus, a HCMV-IEp-hIL2 expression cassette at the ICP47 locus, and an HCMV-IEp-LacZ Pac1 mutating cassette at the gC locus.

10 To create the HX86Z vector, a polynucleotide comprising a gC ICP22 homologous regions and the HCMV-LacZ-IEp-pA mutating cassette were co-transfected into N23 cells with the HX86 vector. Recombinants were screened for LacZ expression, and the correct insertion was confirmed with Southern blot analysis.

#### Expression

15 Expression of each of the transgenes from the multigene HSV vector HX86 was observed.

Expression of the HCMV-IEp-hB7.1 expression cassette at the ICP22 locus was assayed via FACS analysis 24 hours post infection. Depending on MOI, about 50-70% of HX86-infected cells tested positive for hB7.1 expression,  
20 compared to about 5-10% for control cells.

Expression of the HSV-ICP0-Iep-hGM-CSF expression cassette at the UL41 locus was assayed via ELISA analysis 48 hours post infection. Independent of MOI (MOI of 1 and 3 tested), between about HX86-infected cells expressed approximately 210 ng/10<sup>6</sup> cells, compared to less than 50 ng/10<sup>6</sup> cells for all  
25 controls.

Expression of the HCMV-IEp-hIL2 expression cassette at the ICP47 locus was assayed via ELISA analysis 48 hours post infection. At MOIs of 1 and 5, cells infected with HX86 produced 100 and 140 pg hIL2/10<sup>6</sup> cells, respectively, compared to controls which exhibited virtually no hIL2 production.

30 Expression of the HSV-ICP4-IEp-tk expression cassette at the UL24 locus was assayed via gancyclovir selection analysis three days post infection. Uninfected Vero or MCA207 cells exhibited vigorous growth (cell count in millions of cells) both in the presence and absence of 10 µg/ml gancyclovir. Cells infected with HX86, however, exhibited vigorous growth only in the absence of  
35 gancyclovir. Upon treatment with 10 µg/ml gancyclovir, no infected cells, or only a very few infected cells, were present.

# Discussion.

This example demonstrates that the present inventive method can create multigene vectors in which multiple non-native HSV expression cassettes are introduced into a single HSV vector. These results also demonstrate a multigene HSV vector of the present invention comprising four separate expression cassettes, each cassette located in a discrete genetic locus, each cassette expressing a coding polynucleotide in biologically active form, and each cassette expressing its coding polynucleotide under independent and kinetically distinguishable regulatory control.

## WHAT IS CLAIMED IS:

1. A method for preparing a viral vector which comprises:
  - a) co-transfecting a source vector and a mutating cassette together into a population of host cells;  
5 wherein said mutating cassette comprises at least (1) a 3' fragment and a 5' fragment, each of which are homologous to a region of said source vector and (2) a restriction site not present in the sequence of said source vector; and  
whereby homologous recombination occurs between said mutating  
10 cassette and said source vector such that said mutating cassette replaces a genomic polynucleotide within said homologous region;
  - b) plaquing said population of co-transfected host cells;
  - c) selecting plaques comprising cells in which recombination has  
15 occurred between said source vector and said mutating cassette;
  - d) isolating the viral DNA from said plaques;
  - e) digesting said isolated viral DNA with a single restriction endonuclease appropriate for cleaving said viral DNA only at said restriction site within said mutating cassette, whereby two viral  
20 polynucleotides are produced;
  - f) isolating and purifying said two viral polynucleotides; and
  - g) joining said two viral polynucleotides to form a viral vector.
2. The method of claim 1, wherein said mutating cassette comprises two identical restriction sites which flank a region for excision.
- 25 3. The method of claims 1 or 2, wherein said mutating cassette is an expression cassette.
4. The method of any of claims 1-3, wherein said mutating cassette encodes a selectable marker, whereby said recombinant viral DNA is isolated from plaques in which said selectable marker is expressed.
- 30 5. The method of claim 4, in which said selectable marker is  $\beta$ -galactosidase.
6. The method of any of claims 1-5, wherein said source vector is deficient in a native viral locus.
7. The method of any of claims 1-6, wherein said source vector is  
35 replication incompetent.
8. The method of any of claims 1-7, wherein said two viral polynucleotides are joined to form a viral vector comprising said restriction site.



9. The method of any of claims 1-7, wherein said two isolated viral polynucleotides and an insertion cassette are joined to form a viral vector comprising the insertion cassette at the former locus of the restriction site.

5 10. The method of claim 9, wherein said insertion cassette is an expression cassette.

11. The method of claim 10, wherein said insertion cassette encodes RNA.

12. The method of any of claims 1-11, wherein said source vector is an HSV vector.

13. The method of claim 13, wherein said restriction site is PacI.

10 14. An HSV vector constructed in accordance with the method of claims 12 or 13.

15. An HSV vector comprising two or more non-native independent expression cassettes.

15 16. The HSV vector of claim 15, wherein a non-native expression cassette encodes RNA.

17. The HSV vector of any of claims 14-16, which does not require a helper virus for replication.

18. The HSV vector of any of claims 14-17, which expresses tk.

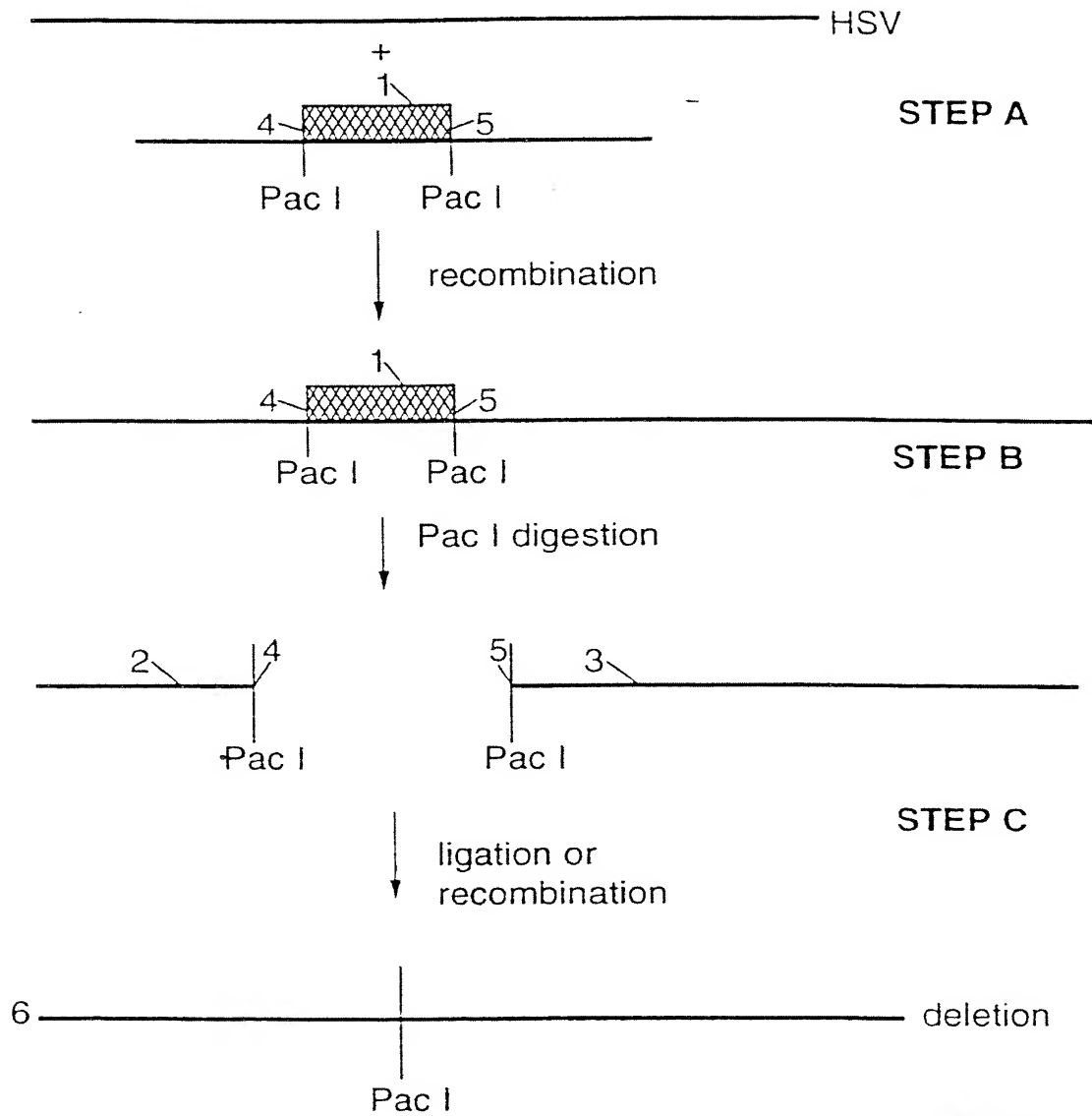
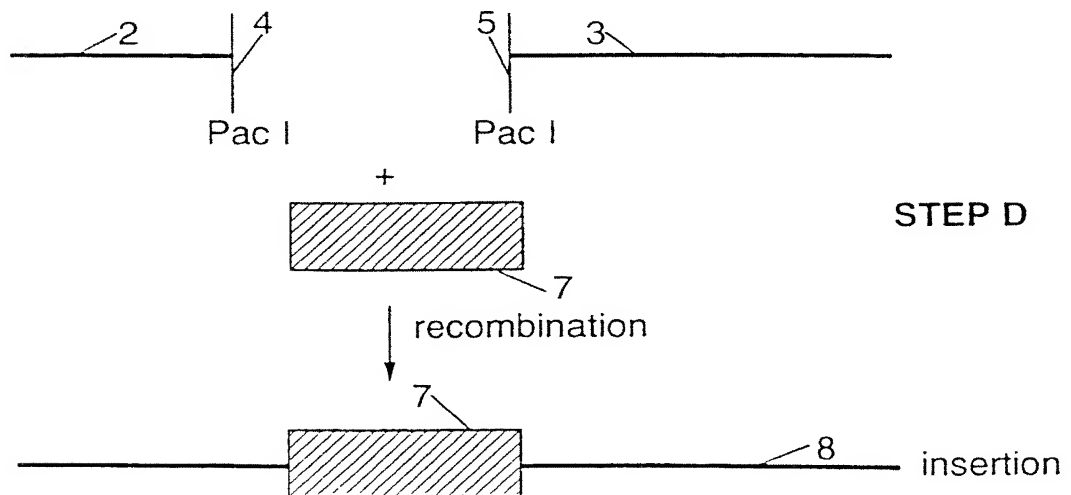
20 19. The HSV vector of any of claims 14-18, which is not deficient for ICP0.

20. The HSV vector of any of claims 15-19, which comprises three or more non-native HSV expression cassettes.

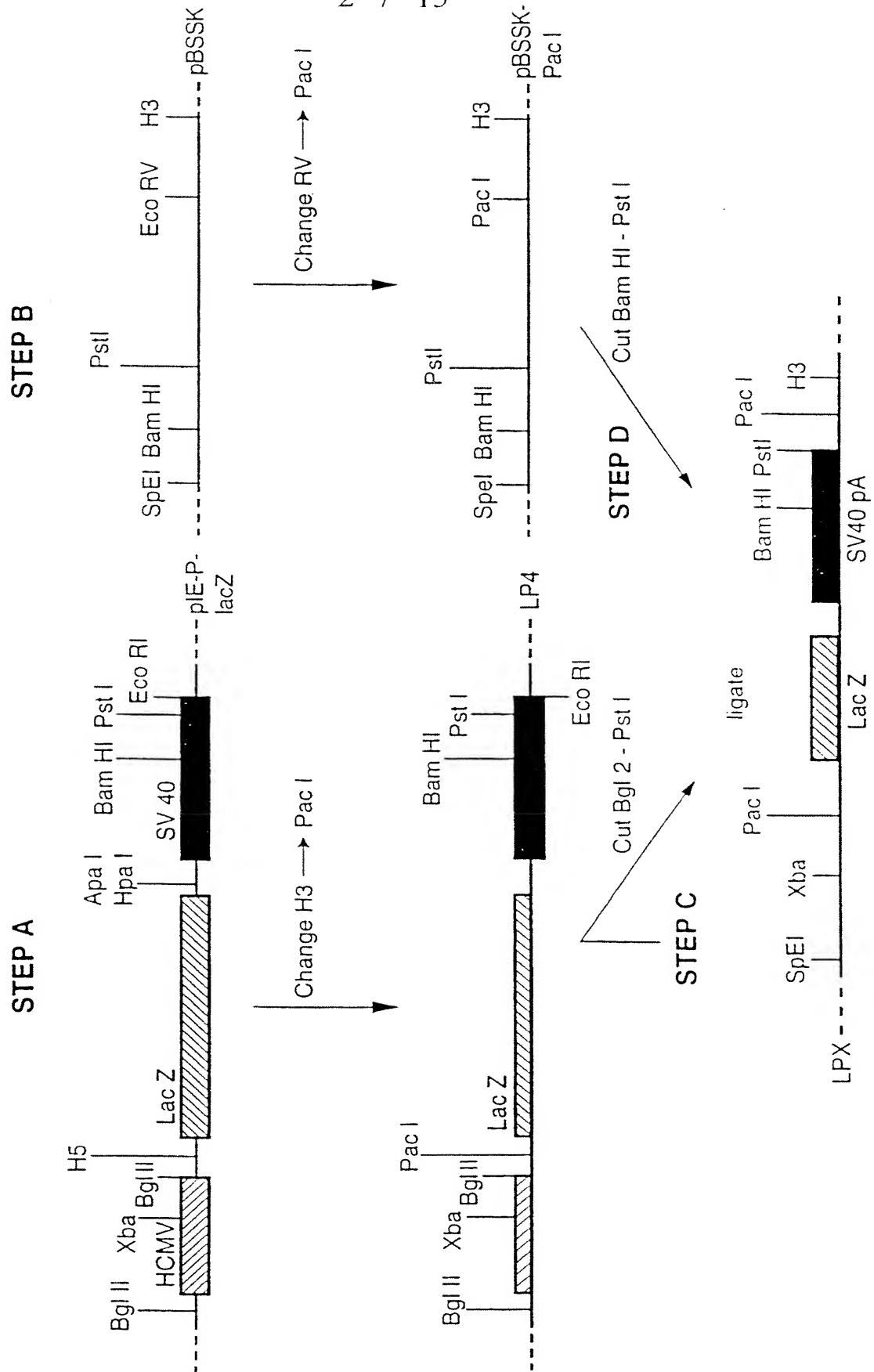
21. The multigene HSV vector of any of claims 15-20, which comprises four or more non-native HSV expression cassettes.

25

1 / 15

**FIG. 1**

2 / 15

**FIG. 2A**

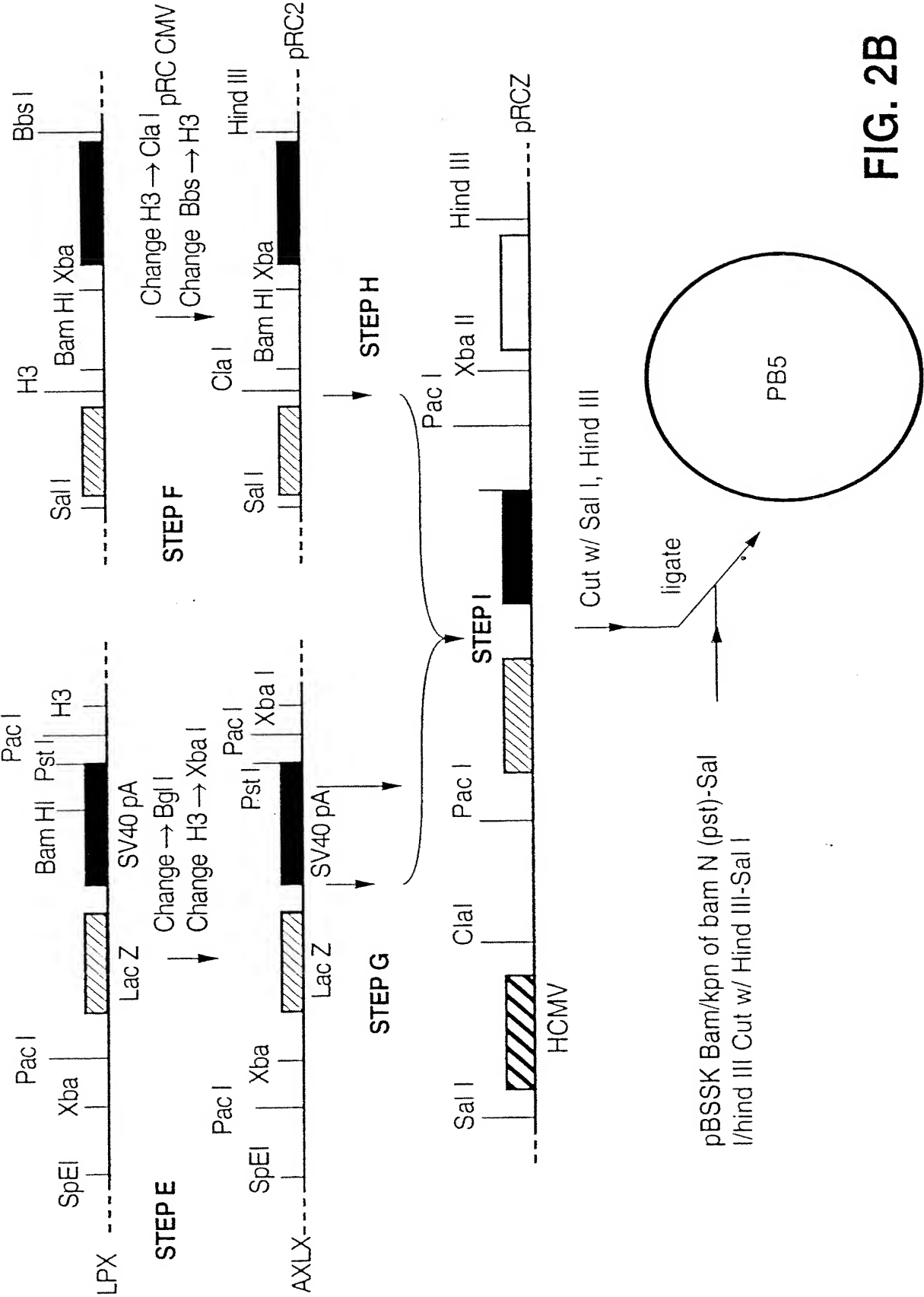
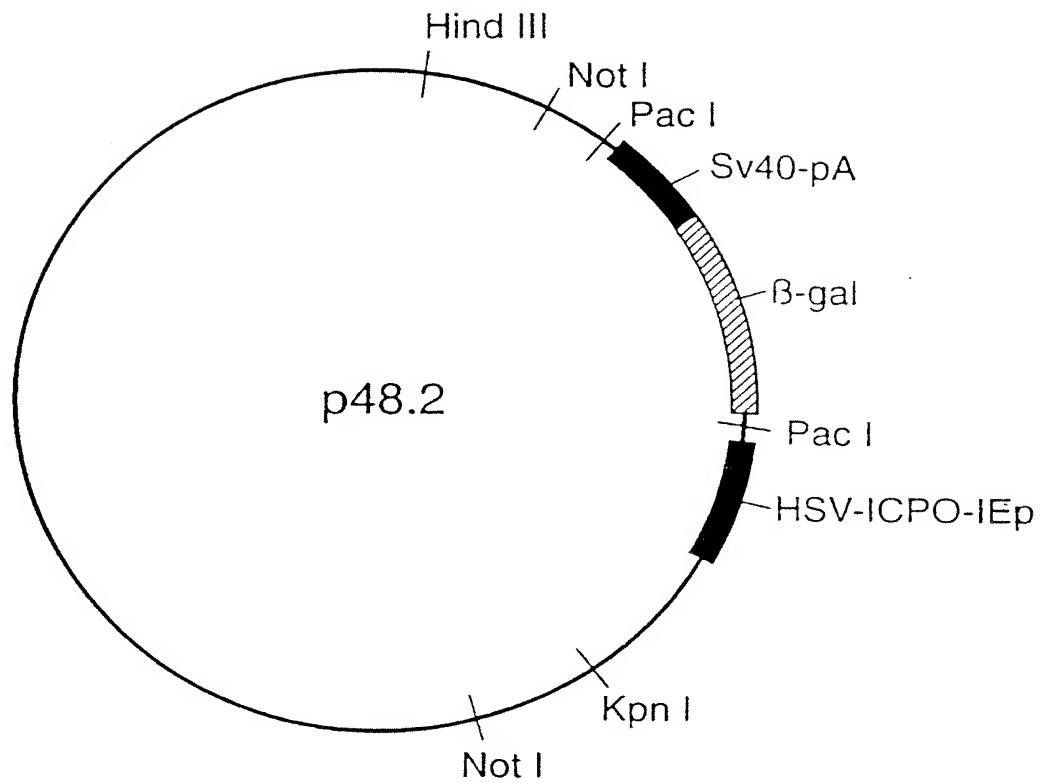
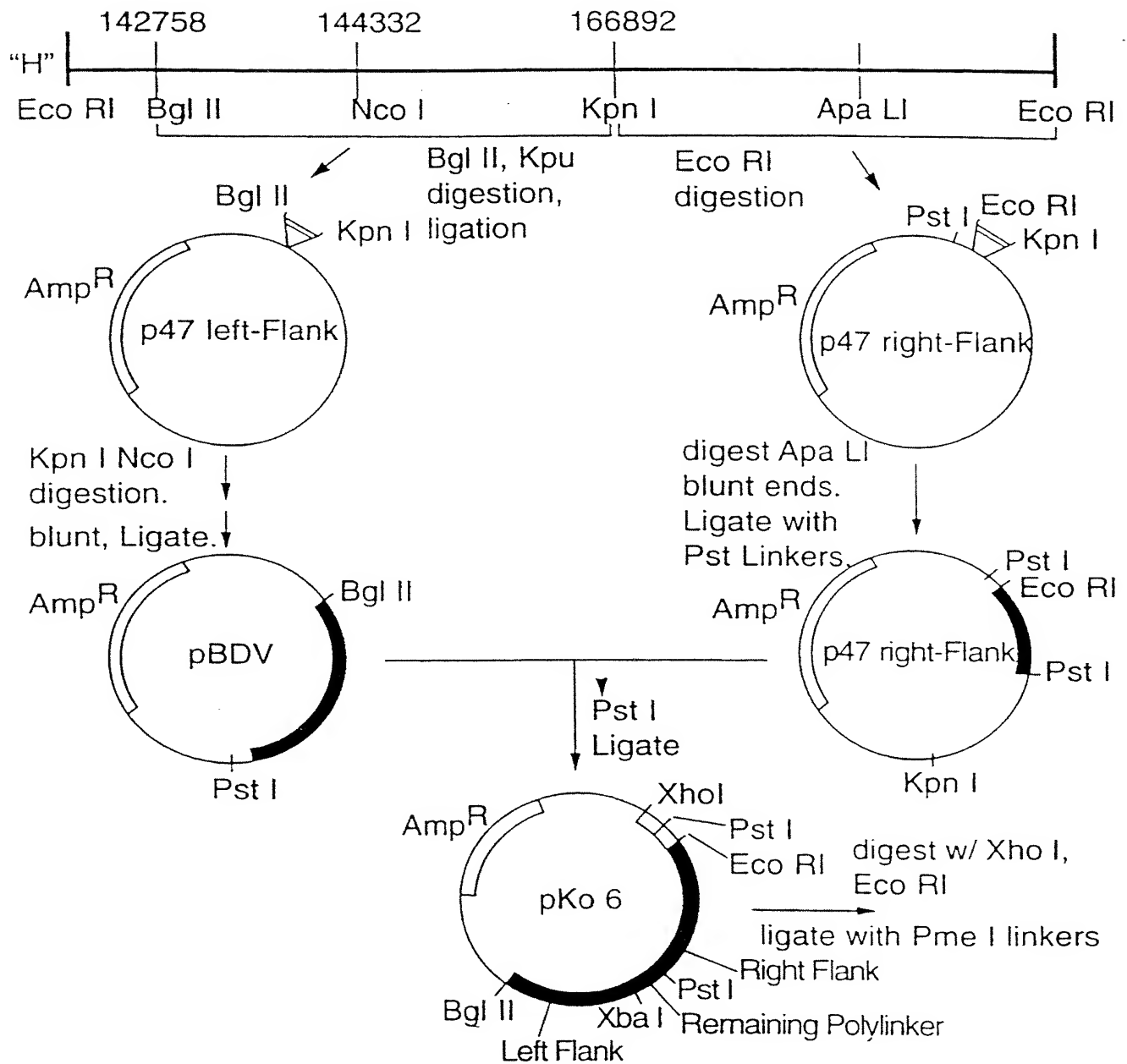
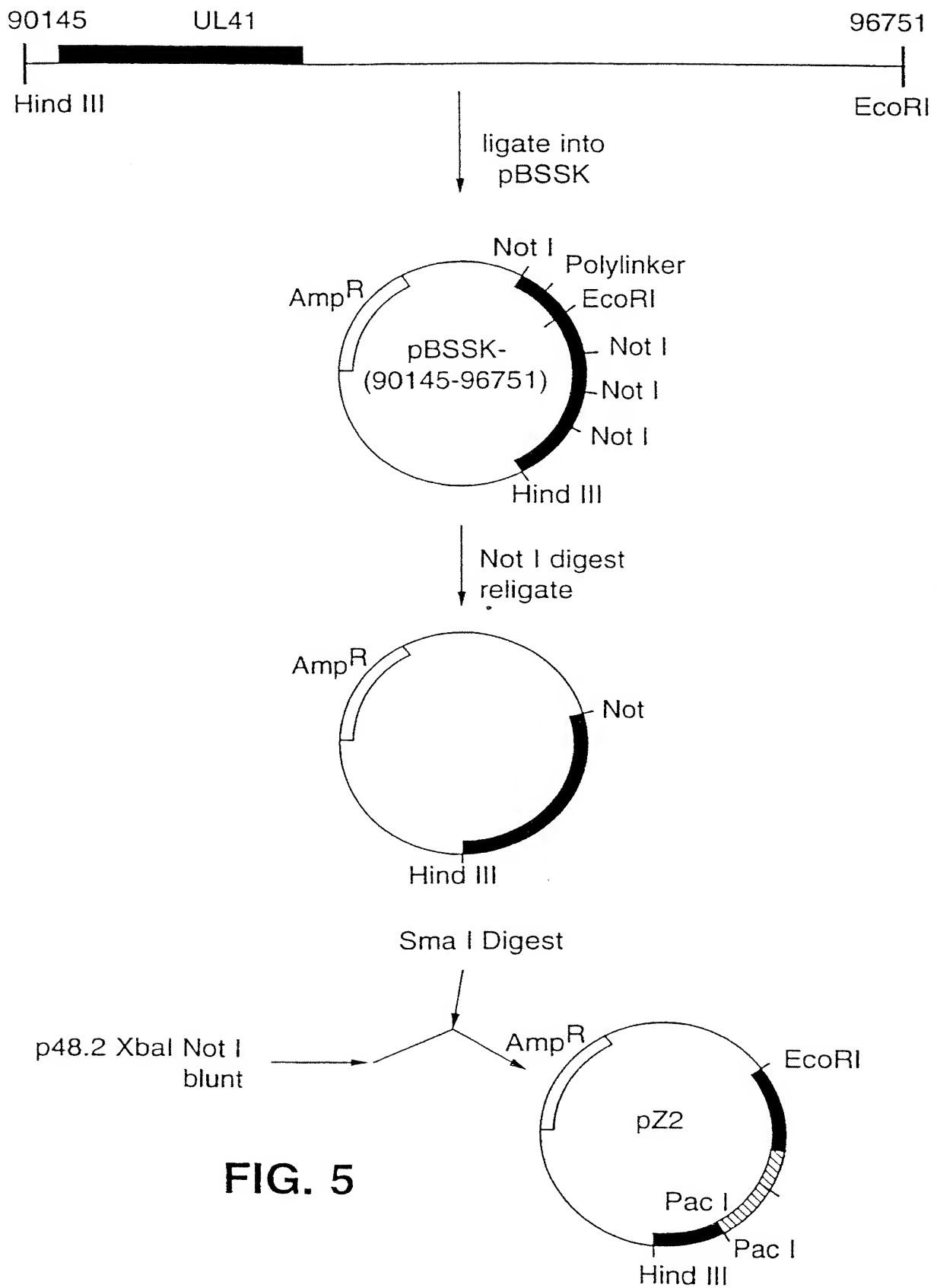


FIG. 2B

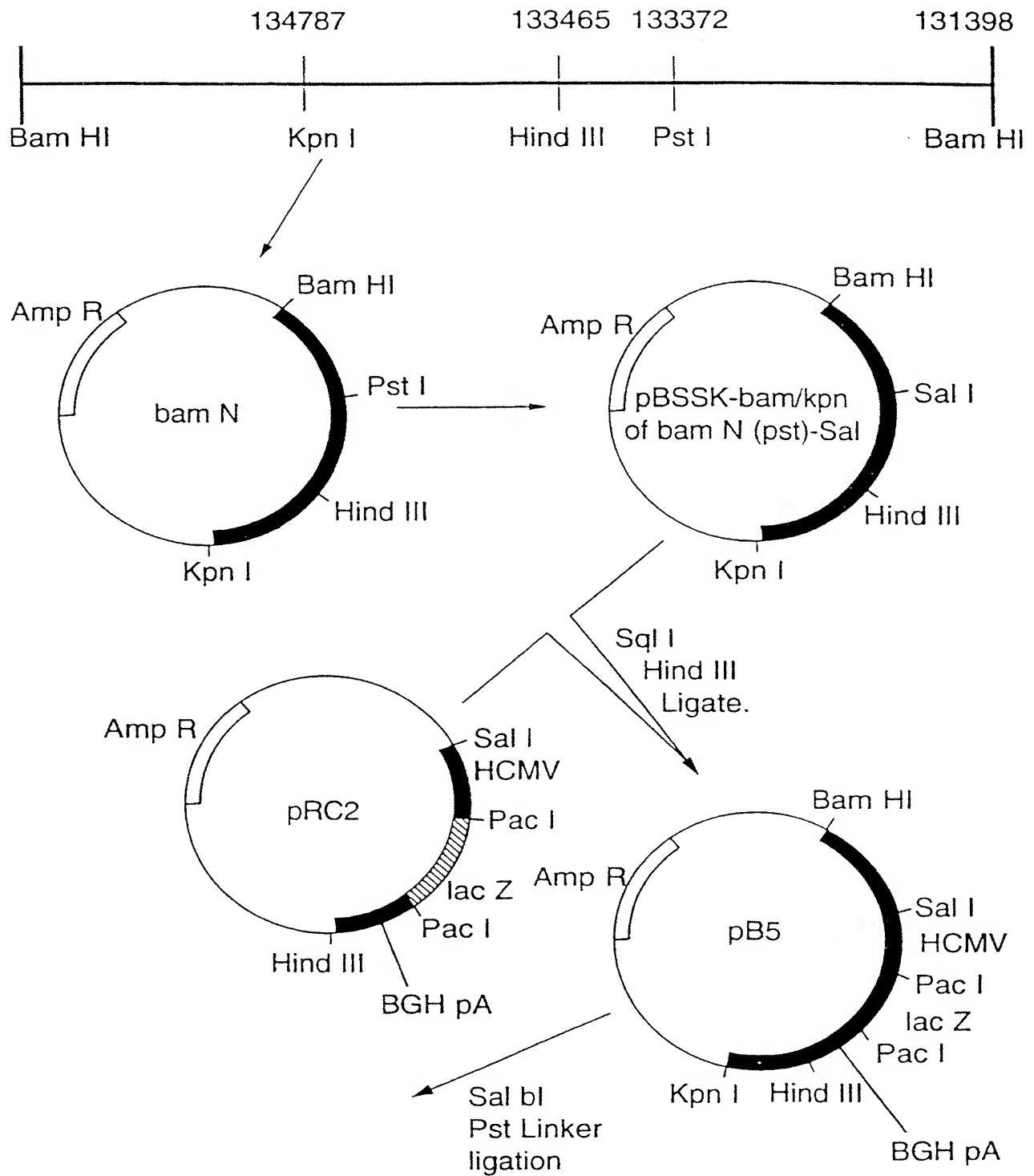
**FIG. 3**

**FIG. 4**

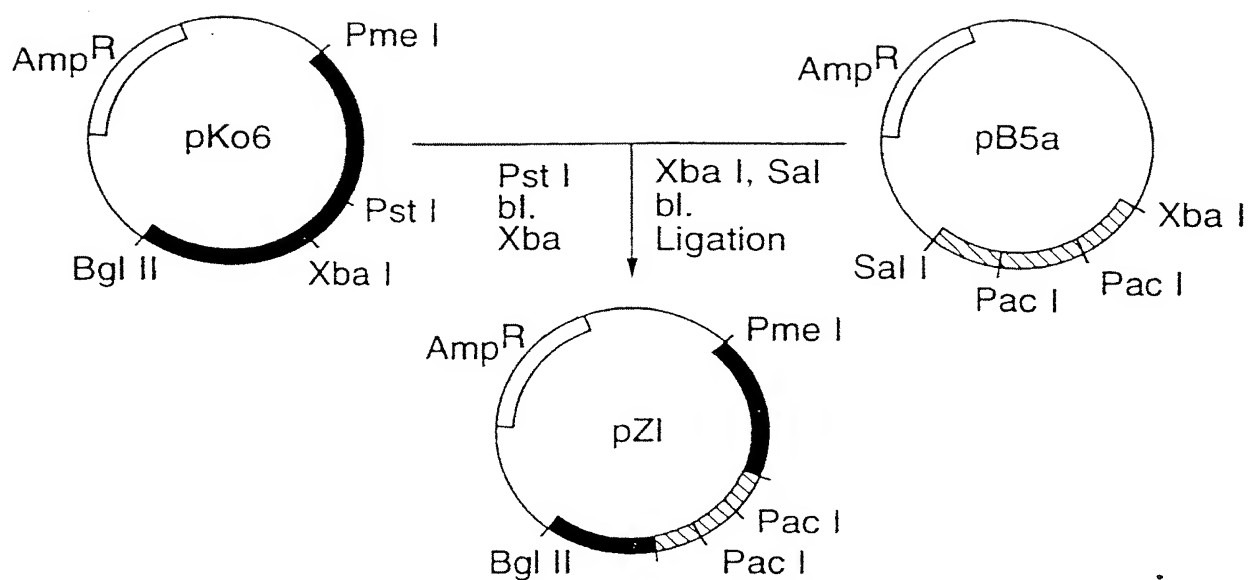
6 / 15

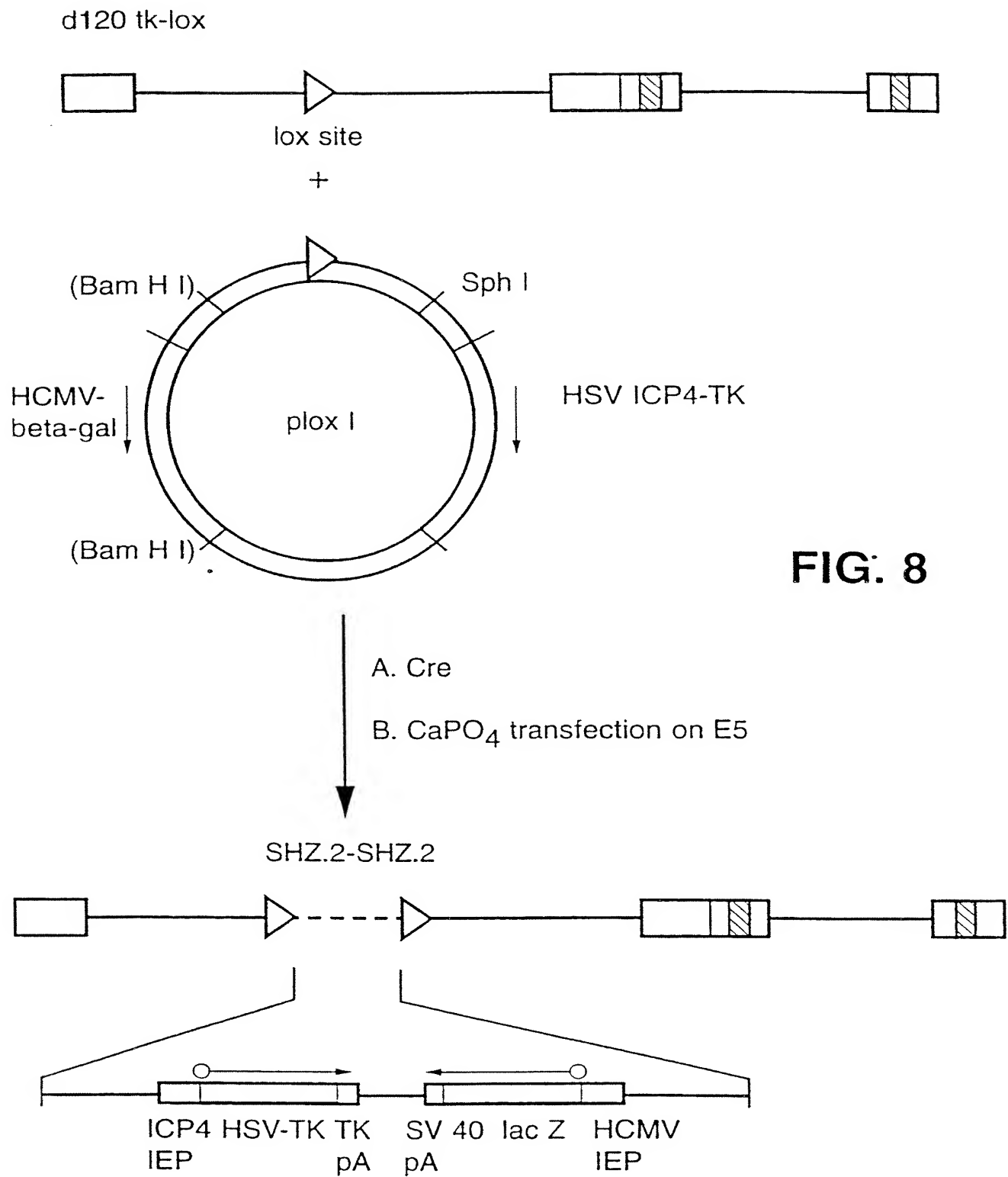
**FIG. 5**

7 / 15

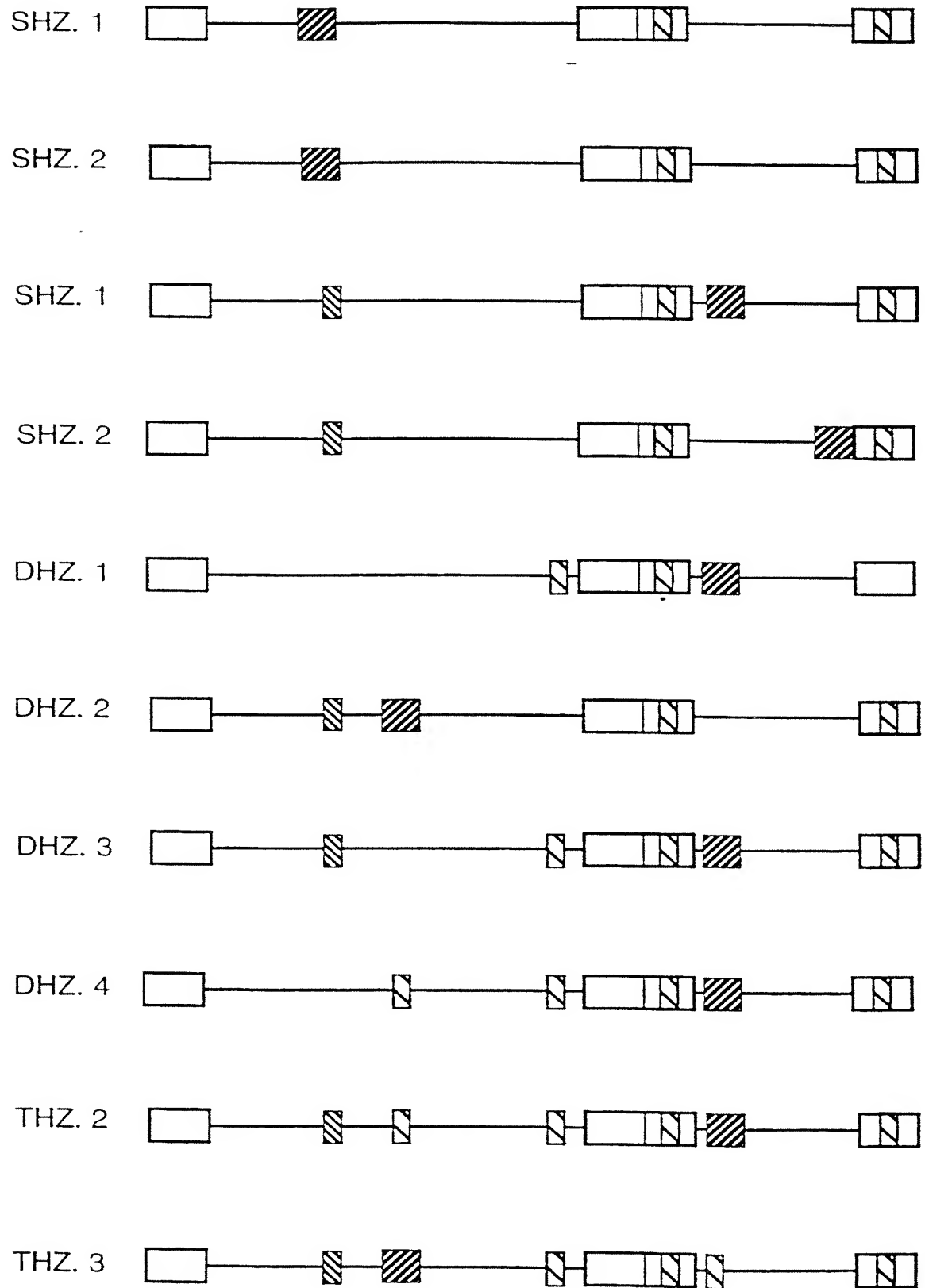
**FIG. 6**

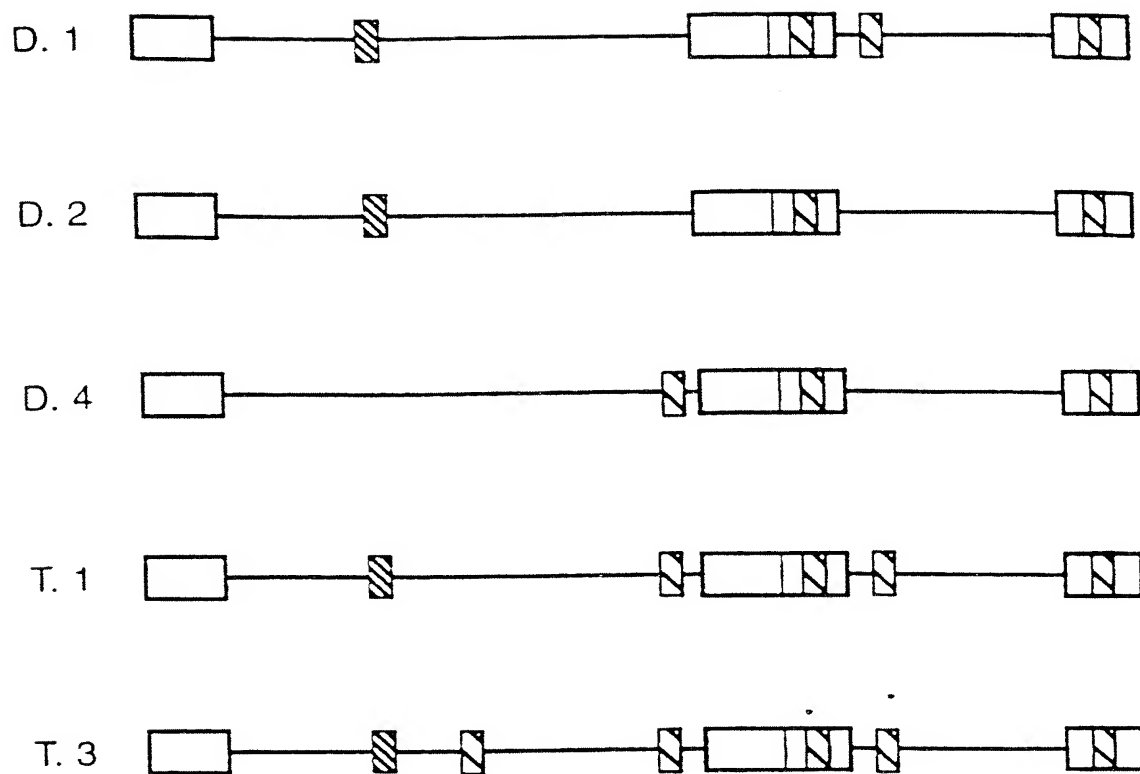


**FIG. 7**



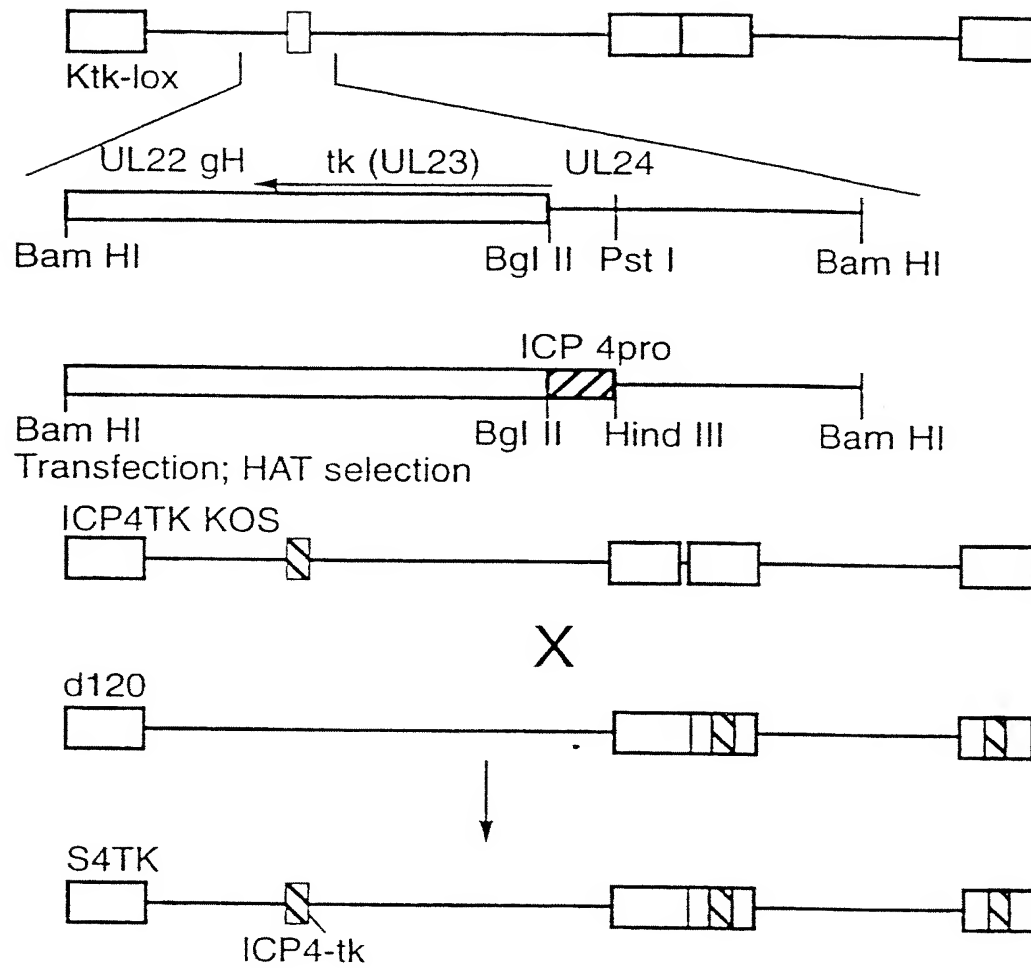
10 / 15

**FIG. 9A**

**FIG. 9B**

12 / 15

STEP A



STEP B

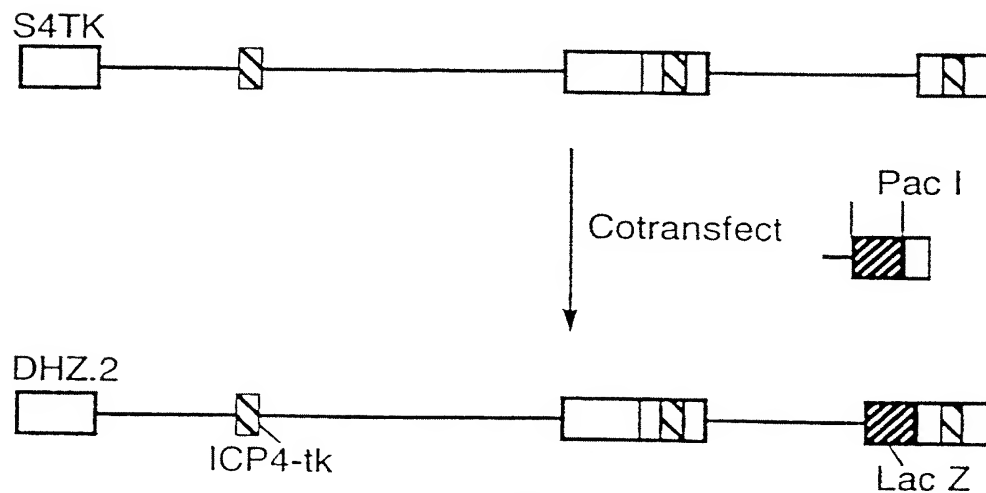
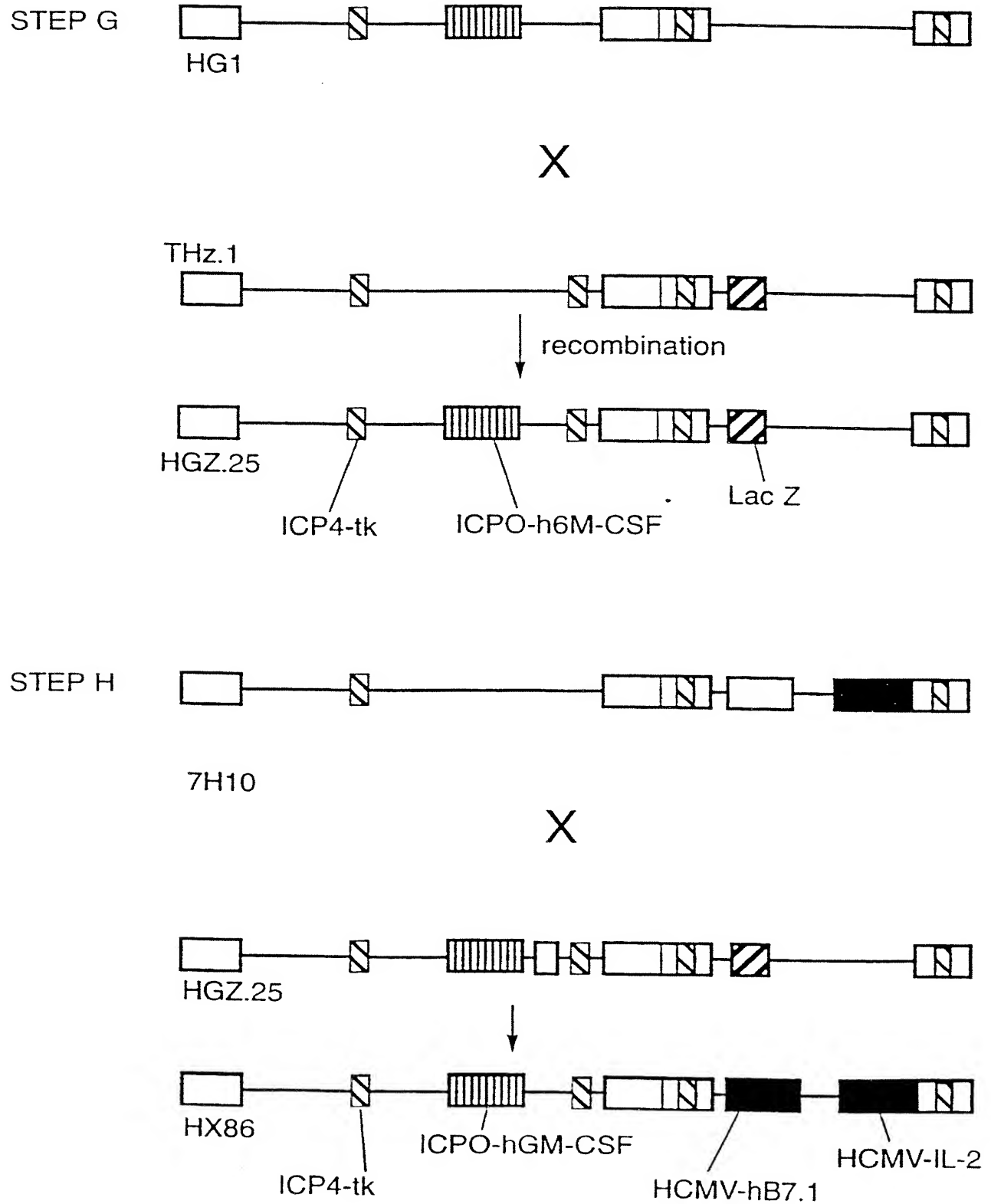


FIG. 10A

**FIG. 10D**

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/09553

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/86

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 04139 A (WISTAR INST) 9 February 1995	1-5, 8-14, 17-19
Y	see the whole document	6,7,15, 16,20,21
Y	GLORIOSO J. C. ET AL.: "GENE TRANSFER TO BRAIN USING HERPES SIMPLEX VIRUS VECTORS" ANNALS OF NEUROLOGY, vol. 35, 1994, pages S28-S34, XP000672795 see the whole document	6,7,15, 16,20,21

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

6 October 1998

Date of mailing of the international search report

16/10/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Mandl, B

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUANG Q. S. ET AL.: "An in vitro ligation and transfection system for inserting DNA sequences into the latency-associated transcripts (LATs) gene of herpes simplex virus type 1" GENE THERAPY, vol. 5, no. 1, 1994, pages 300-306, XP002079294 cited in the application see the whole document	1-5, 8-14, 17-19
X	WO 97 14808 A (ST JUDE CHILDRENS RES HOSPITAL ;CANTAB PHARMA RES (GB); BOURNSNELL) 24 April 1997 see the whole document, especially page 28, lines 17-30 and page 32, lines 15-29	1,6-14, 17-19
X	RIXON F. J. AND MCLAUCHLAN: "Insertion of DNA sequences at a unique restriction enzyme site engineered for vector purposes into the genome of herpes simplex virus type 1." JOURNAL OF GENERAL VIROLOGY, vol. 71, 1990, pages 2931-2939, XP002079295 cited in the application see the whole document	1,8-14, 17-19
A	ROIZMAN B. AND JENKINS F. J.: "Genetic engineering of novel genomes of large DNA viruses." SCIENCE, vol. 229, 1985, pages 1208-1214, XP002079296 see the whole document	1-21
A	GAGE P. J. ET AL.: "A CELL-FREE RECOMBINATION SYSTEM FOR SITE-SPECIFIC INTEGRATION OF MULTIGENIC SHUTTLE PLASMIDS INTO THE HERPES SIMPLEX VIRUS TYPE 1 GENOME" JOURNAL OF VIROLOGY, vol. 66, no. 9, September 1992, pages 5509-5515, XP000674658 cited in the application	1-21
A	LEIB D. A. AND OLIVO P. D.: "Gene delivery to neurons: Is herpes simplex virus the right tool for the job?" BIOASSAYS, vol. 15, no. 8, 1993, pages 547-554, XP002079297 see page 550, left-hand column; figure 2A	1-21

-/--



## INTERNATIONAL SEARCH REPORT

Int. national Application No.

PCT/US 98/09553

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 04394 A (MEDICAL RES COUNCIL ;UNIV DUNDEE (GB); PRESTON CHRISTOPHER MAURICE) 15 February 1996 see page 8, column 2 - page 11, column 1 ---	1-21
P,X	KRISKY D. M. ET AL.: "Rapid method for construction of recombinant HSV gene transfer vectors." GENE THERAPY, vol. 4, no. 10, October 1997, pages 1120-1125, XP002079298 see the whole document -----	1-21

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 09553

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
Claim 13 was read and searched as: 'The method of claim 12, wherein....'.
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9504139 A	09-02-1995	CA 2167706 A EP 0722493 A JP 9501564 T	09-02-1995 24-07-1996 18-02-1997
WO 9714808 A	24-04-1997	AU 7315196 A EP 0856062 A	07-05-1997 05-08-1998
WO 9604394 A	15-02-1996	AU 695405 B AU 3119595 A CA 2195965 A EP 0804602 A JP 10503372 T	13-08-1998 04-03-1996 15-02-1996 05-11-1997 31-03-1998

